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

O-ACYLATION OF SIALIC ACIDS IN THE RAT LIVER GOLGI APPARATUS INVOLVES AN ACETYL INTERMEDIATE AND ESSENTIAL HISTIDINE AND LYSINE RESIDUES—A TRANSMEMBRANE REACTION*

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Isolated intact rat liver Golgi vesicles utilize [acetyl-3H]coenzyme A to add 3H-O-acetyl esters to sialic acids of internally facing endogenous glycoproteins. During this reaction, [3H]acetate also accumulates in the vesicles, even though the vesicles are impermeant to free acetate. On the other hand, entry of intact AcCoA into the lumen of the vesicles could not be demonstrated, and permeabilization of the vesicles did not alter the reaction substantially (Diaz, S., Higa, H. H., Hayes, B. K., and Varki, A. (1989) J. Biol. Chem. 264, 19416–19426). When vesicles prelabeled with [acetyl-3H]coenzyme A are permeabilized with saponin, we can demonstrate a [3H]acetate intermediate in the membrane that can transfer label to the 7- and 9-positions of exogenously added free N-acetylneuraminic acid but not to glucuronic acid or CMP-N-acetylneuraminic acid. This labeled acetyl intermediate represents a significant portion of the radioactivity incorporated into the membranes during the initial incubation and cannot be accounted for by nonspecifically "trapped" acetyl-CoA in the permeabilized vesicles. There was no evidence for involvement of acetylcarnitine or acetyl phosphate as an intermediate. The overall acetylation reaction appears to involve two steps. The first step (utilization of exogenous acetyl-CoA to form the acetyl intermediate) is inhibited by coenzyme A-SH (apparent $K_i = 24–29 \mu M$), whereas the second (transfer from the acetyl intermediate to sialic acid) is not affected by millimolar concentrations of the nucleotide. Studies with amino acid-modifying reagents indicate that 1 or more histidine residues are involved in the first step of the acetylation reaction. Diethylpyrocarbonate (which can react with both nonsubstituted and singly acetylated histidine residues) also blocks the second reaction, indicating that the acetyl intermediate on both sides of the membrane involves histidine residue(s). Taken together with data presented in the preceding paper, these results indicate that the acetylation of sialic acids in Golgi vesicles may occur by a transmembrane reaction, similar to that described for the acetylation of glucosamine in lysosomes (Bame, K. J., and Rome, I. H. (1985) J. Biol. Chem. 260, 11293–11299). However, several features of this Golgi reaction distinguish it from the lysosomal one, including the nature and kinetics of the reaction and the additional involvement of an essential lysine residue. The accumulation of free acetate in the lumen of the vesicles during the reaction may occur by abortive acetylation (viz. transfer of label from the acetyl intermediate to water). It is not clear if this is an artifact that occurs only in the in vitro reaction.

A wide variety of biological reactions involving the transfer of an acetyl group are known. In most of these reactions, the donor is acetyl coenzyme A (AcCoA), with acetyl intermediates such as acetylcarnitine playing an important role in some cases (1–10). In preceding work, we described studies of the O-acetylation of sialic acids in isolated intact rat liver Golgi vesicles (11, 12). Several questions have arisen from this work. Since intact [3H]AcCoA does not appear to accumulate in the lumen of the vesicle, how is it that transfer of [3H]acetyl groups to internally facing sialic acids is effected? Why is it that following solubilization of the vesicles with nonionic detergents, the O-acetyltransferase activity can no longer be detected using exogenous acceptors? Since the vesicles are impermeant to free acetate, how is it that [3H]acetate accumulates in the intact vesicles, with kinetics suggesting it is a product of abortive acetylation? On the other hand, how is it that acetylation can proceed almost unchanged after permeabilization of the vesicles to low molecular weight substances? We reasoned that an O-acetylation mechanism in which acetyl groups are transferred across the membrane via a [3H]acetyl intermediate would provide the best answer for all of these questions. In this study, we provide evidence for such a mechanism, involving a probable histidine active site residue and an essential lysine. The mechanism appears to be similar, but not identical, with that described for the acetylation of glucosamine in the lysosomes by Rome and others (10, 13–15).

**EXPERIMENTAL PROCEDURES**

Some of the materials, methods, and procedures used in this study are similar to those in the preceding paper (12) and will not be listed here again. Others are described below in detail.

**Materials**

The following materials were obtained from Sigma: acetylcarnitine, carnitine, and acetyl phosphate, diethyl pyrocarbonate (DEP), N-
bromosuccinimide (NBS), N-acetylneurimidozole, succinic anhydride, N-ethylmaleimide, iodoacetamide, methylamine, p-chloromercuribenzoate, and trinitrobenzenesulfonic acid (TNBS). Dibisopropyl fluorophosphate was obtained from Aldrich and prepared as 1 M and 100 mM stocks in isopropyl alcohol, which were stored in glass screw-cap vials in a desiccator at -20°C.

Methods

Amino Acid-modifying Reagents—Stock solutions of the reagents were prepared as follows. DEP: 34 mM in ethanol; NBS: 0.5 M in acetonitrile, made 10 mM in water just prior to use; N-acetylneuraminidazole: 100 mM in water, made fresh; succinic anhydride: stock solution 1.66 M in acetonitrile, 10 mM prepared fresh in water before use; N-ethylmaleimide: 10 mM in water, made fresh; iodoacetamide: 10 mM in water, made fresh; methylamine: 100 mM in water; p-chloromercuribenzoate: 45 mM in 100 mM NaOH; trinitrobenzenesulfonic acid: 10 mM in 10 mM KPi, pH 8.0. All reactions with the Golgi vesicles were carried out at pH 6.5, except for TNBS which was incubated at pH 8.0 (with appropriate controls).

Descending Paper Chromatography—This was carried out on Whatman No. 3MM paper in 95% ethanol:1 M NH4OAc, pH 5.3 (79:21), for 12-14 h. Schilling Green Food Coloring (McCormick and Co.) was co-spotted with samples (15 μl of a 50% solution per lane). The color separates into two spots (blue and yellow) during chromatography, which provide internal markers for the progress of the chromatography and minor variations between lanes.

HPLC Systems

System A—Acetyl-CoA and acetate were separated with an Alltech Versapack C-18 column (250 × 4.1 mm) run in the isocratic mode at 1 ml/min with 50% methanol:water:0.25 M NaH2PO4 (18:66:18). As the column ages, the final percent of methanol requires reduction to maintain the same separation.

System B—The different sialic acids were separated using a Bio-Rad HPX-72S column (300 × 7.8 mm) eluted in the isocratic mode at 1 ml/min with 100 mM Na2SO4 (12).

Buffers—The buffers used were: PK buffer, 10 mM potassium phosphate, pH 6.5, 150 mM KCl; PKM buffer, 10 mM potassium phosphate, pH 6.5, 150 mM KCl, 1 M MgCl2.

Assay for Transfer of Acetyl to Free Sialic Acids from the Membrane Acetyl Intermediate

Golgi vesicles (5 mg) were first labeled with [3H]AcCoA (13.5 μCi) in 1 ml of PK or PKM buffer for 20 min at 22°C. A small portion (<10%) of the membrane-associated radioactivity is in the form of a [3H]acetyl intermediate that can be transferred to free sialic acid. The initial labeling reaction was quenched with 3.0 ml of PK buffer containing 0.1% saponin and centrifuged at 100,000 g for 30 min at 4°C. The supernatant was aspirated off, and the pellet was carefully surface-washed twice with 4 ml of cold PK buffer containing 0.1% saponin. One ml of the same buffer was added to the pellet, allowed to stand on ice for 5-30 min, and aspirated off carefully. The pellet was resuspended in 170 μl of PK buffer by brief sonication, just sufficient to resuspend the vesicles (excessive sonication results in substantial loss of activity). Aliquots (40 μl) of the suspension were incubated in 100-μl reactions containing 10 mM Neu5Ac2 (sample) or 10 mM glucuronic acid (GluA) (control) in PK buffer. The reactions (in ultracentrifuge tubes) were incubated at 25°C for 30 min and centrifuged at 100,000 × g for 30 min. 100 μl of the supernatant was transferred into 900 μl of ice-cold 100% ethanol in Eppendorf tubes, mixed, and placed on ice for 1-2 h. The tubes were centrifuged at 10,000 × g for 15 min at 4°C. The supernatants were transferred into fresh tubes and dried on a Savant Speed-Vac. The samples were analyzed by descending paper chromatography as described above. The lanes were cut into 1-cm strips and soaked in 0.5 ml of water, 5 ml of scintillation cocktail was added, and the radioactivity was determined. The product (a mixture of [3H]Neu5,9Ac2 and [3H]Neu5,7Ac2) was found in a single peak with an Rf of 0.7, immediately behind the blue marker spot. This peak was not seen in the GluA control, and the background from this lane was subtracted. The GluA control, and the background from this lane was subtracted. When necessary, an internal standard of [14C]Neu5Ac (2,000 cpm) was also added to each sample before spotting to further monitor

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Phosphate in the Transfer Reaction—The results presented in the preceding paper (12) suggest that the acetylation reaction does not involve the transport of intact acetyl-CoA into the lumen of the vesicles. This raised the possibility of a transmembrane acetylation reaction. We first investigated the involvement of an acetylcarbonyl intermediate or a high energy acetyl phosphate intermediate in such a reaction. We found that the addition of carnitine, acetylcarnitine, or acetyl phosphate at concentrations as high as 1 mM had little effect upon the utilization of the [acetyl-3H]AcCoA by the Golgi vesicles (data not shown). This indicates that the involvement of these intermediates in this reaction is unlikely, at least in the first step of the reaction (1). We therefore looked for an alternate mechanism for mono-O-acetylated sialic acids. It was also seen from this chromatogram that there is insufficient [acetyl-3H]AcCoA "trapped" in the vesicles to account for the generation of the O-acetylated sialic acids by an alternate mechanism. This point was confirmed and quantitated by repeating the experiment with a careful monitoring of recovered radioactivity. [acetyl-3H]AcCoA-labeled, saponin-washed Golgi membranes (215,625 cpm each) were incubated with ethanol as described under "Experimental Procedures," either immediately or after a 30-min incubation with 10 mM GluA or 10 mM Neu5Ac at 25°C. The ethanol supernatants were dried down and analyzed by paper chromatography as above. The amount of residual radioactivity in the [acetyl-3H]AcCoA region of the chromatogram at zero time was 940 cpm, and no significant change was seen in the incubated samples. The amount of radioactivity that ran in the region of O-acetylated sialic acids was 8854 cpm with the Neu5Ac and 2750 cpm with the control GluA incubation. Thus, it can be calculated that the 6104 cpm of specific product could not have been derived from the 940 cpm of transferable [acetyl-3H]AcCoA. It can also be calculated from this experiment that the [3H]acetyl intermediate accounts for at least 2.8% of the total saponin-resistant membrane-associated radioactivity. This actually represents an underestimate, since the reaction continues in a linear fashion for up to 3 h (not shown), and the 10 mM Neu5Ac used is not saturating (see below).

Proof of the Products of the Transfer Reaction—The Neu5Ac-dependent peak of radioactivity was prepared by
elution from a paper chromatogram after a preparative scale reaction (not shown). Analysis of this radioactivity by HPLC showed that its elution pattern was that of a mixture of 7- and 9-O-[3H]acetyl-Neu5Ac, the expected products of the exchange reaction with an acetyl intermediate (see upper panel of Fig. 2). As shown in the remaining panels of Fig. 2, the nature of these molecules was further confirmed by treatments with mild alkali (migration of the acetyl groups occurred from the 7- to the 9-position), strong alkali (de-0-acetylation to free acetate), and with a sialate-specific 9-O-acetylerase purified from rat liver (16) (enzymatic de-0-acetylation). In each case, the great majority of the radioactivity behaved exactly as predicted for a mixture of 7- and 9-O-[acetyl-3H]acetyl-Neu5Ac.

Comparison of Transfer to Free Sialic Acid and CMP-Sialic Acid—In the above experiment, transfer of radioactivity from the intermediate was shown to be specific for Neu5Ac, but not GluA. To further demonstrate the specificity of the transfer reaction for Neu5Ac, we compared the acceptor activity of the free sugar with that of the β-linked sugar nucleotide, CMP-Neu5Ac. Prelabeled membranes were washed and permeabilized, incubated with GluA, Neu5Ac, or CMP-Neu5Ac, and the ethanol-soluble products of the reaction were analyzed by paper chromatography as described above. As shown in Table I, we found that transfer to the sugar nucleotide from the [3H]acetyl intermediate was barely detectable under conditions where substantial transfer to the free sugar occurred. In fact, free Neu5Ac released from partial breakdown of CMP-Neu5Ac during the incubation was a much better acceptor than the remaining sugar nucleotide in the very same reaction mixture. These results corroborate well with those presented in the preceding paper, in which we found that labeled CMP-[3H]Neu5Ac added to intact Golgi vesicles was not significantly acetylated, even in the presence of added unlabeled acetyl-CoA.

Comparison of the Properties and Kinetics of the Two Half-Reactions—The overall acetylation reaction thus could involve two steps. The first step would involve utilization of exogenous acetyl-CoA to form the acetyl intermediate, whereas the second would involve transfer from the acetyl intermediate to sialic acid. The assay described above could be used to study the second component of the acetylation reaction. However, in order to study the kinetics of the first component (generation of the acetyl intermediate) in isolation, it is necessary to eliminate the endogenous acceptor substrate prior to labeling. All attempts to do so were unsuccessful, presumably because the substrate is present in a protected environment (an intact vesicle). Neuraminidase treatments at moderate concentrations of saponin were unsuccessful in destroying the substrates. Mild periodate treatment destroyed all activity with or without low concentrations of saponin, as did higher concentrations of saponin alone. Thus, the kinetics of the first half-reaction could not be studied in isolation. Instead, we compared the kinetics of the overall reaction (acetylation of endogenous sialic acids in intact vesicles) with the second half-reaction (transfer from the acetyl intermediate in permeabilized vesicles to exogenously added sialic acid).

In the preceding paper (12), we reported the kinetics of the overall reaction for AcCoA (apparent $K_m = 2.9 \mu M$, $V_{max} = 45$ pmol/min/mg of protein). The kinetics of the second half-reaction for Neu5Ac was studied using the paper chromatography assay described above. This reaction shows an apparent $K_m$ of 13.9 mM for Neu5Ac, with a $V_{max}$ of 0.57 pmol/min/mg of protein (data not shown). Thus, the transfer to endogenous glycoprotein acceptors by the complete system appears to be far more efficient than that from the acetyl intermediate to the exogenously added free sugar. The pH/activity profiles of these two reactions are presented in Fig. 3. It can be seen that the two reactions had slightly different profiles. The overall reaction had a neutral pH optimum, whereas the second half-reaction occurred better at a slightly acidic pH. Furthermore,
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Fig. 2. Proof of product of the second transfer reaction. A scaled up transfer reaction similar to that described in the legend to Fig. 1 was performed in the presence of 10 mM Neu5Ac. One-cm strips of the paper chromatogram were cut and soaked in 1 ml of 10 mM acetic acid, and the radioactivity was monitored. The Neu5Ac-specific peak was pooled, dried on a shaker evaporator, and analyzed by HPLC on a HPX-72-S column with or without the various treatments indicated. The position of elution of standards are indicated.

TABLE I
Comparison of transfer from the [\(^{14}\)C]acetyl intermediate to sialic acid and CMP-sialic acid

Golgi vesicles were prelabeled with [\(^{14}\)C]AcCoA, permeabilized, and washed to prepare the [\(^{14}\)H]acetyl intermediate, exactly as described in the legend to Fig. 2. The labeled membranes were incubated in duplicate with 10 mM Neu5Ac, CMP-Neu5Ac, or GluA in PK buffer, pH 5.5, at 25°C for 3 h. The ethanol-soluble products of the reaction were analyzed by paper chromatography as described under "Experimental Procedures." In each lane, the amount of \(^3\)H radioactivity migrating in the position of the CMP-sialic acids and that running in the position of free sialic acid was calculated. The results presented are the mean values obtained, after subtraction of the nonspecific background seen in the GluA incubations. An internal standard of CMP-[\(^{14}\)C]Neu5Ac was monitored for breakdown of the nucleotide sugar. This showed a mean of 41% residual nucleotide sugar at the end of the reaction, with 51% of the label migrating as free sialic acid.

<table>
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<th>Acceptor</th>
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<th>(\text{cpm})</th>
</tr>
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<tr>
<td></td>
<td>CMP-sialic acids</td>
<td>Free sialic acids</td>
</tr>
<tr>
<td>CMP-Neu5Ac (10 mM)</td>
<td>73</td>
<td>376</td>
</tr>
<tr>
<td>Neu5Ac (10 mM)</td>
<td>&lt;10</td>
<td>570</td>
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</table>

Fig. 3. Comparison of the pH/activity profiles of the two reactions. Golgi vesicles were studied for the incorporation of label from [acetyl-\(^3\)H]AcCoA into acid-insoluble materials (overall reaction) exactly as described in the legend to Fig. 1, except that the incubations were performed in the various buffers (acetate, closed square; phosphate, open square; citrate-phosphate, closed triangle; and Tris-HCl, open triangle; all at 100 mM final) at the pH values indicated (upper panel). Golgi vesicles were prelabeled with [acetyl-\(^3\)H]AcCoA, permeabilized, washed, and studied for the transfer of label to 10 mM Neu5Ac (second half-reaction) exactly as described in the legend to Fig. 2, with the incubations being performed in the various buffers and pH values as indicated (lower panel). Appropriate blank values were subtracted (see "Experimental Procedures"). The results are presented as a percentage of the maximum values obtained in each case (26,885 cpm for the combined reaction and 5,004 cpm for the second reaction).

The second reaction was partially inhibited by acetate buffer, whereas the overall reaction was not.

We next studied the effects of coenzyme A-SH upon the two reactions. We found that the overall reaction was substantially inhibited by CoA-SH, with half-maximal inhibition at about 30 \(\mu\)M. More detailed kinetic studies were carried out to obtain inhibition constants. As in all previous experiments in this system, the results obtained were nearly identical regardless of whether one considers the acid-soluble (acetate) and acid-insoluble (O-acetylated sialic acids) components as separate products, or as the combined products of a single reaction. The data presented in Fig. 4 are for the acid-insoluble component only. They show a pattern consistent with competitive inhibition and a \(K_i\) of 26.3 \(\mu\)M. The \(K_i\) obtained using the acid-soluble component was 23.8 \(\mu\)M, and that obtained by combining both products was 29.4 \(\mu\)M (data not shown). In each case, the pattern also indicated competitive inhibition (data not shown). On the other hand, we found that the second reaction (transfer from the acetyl
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![Graph showing the kinetics of inhibition of the overall reaction by CoA.](image)

**Fig. 4. Example of the kinetics of inhibition of the overall reaction by CoA.** Golgi vesicles (0.24 mg of protein) were incubated with \(^{3}H\)AcCoA (0.24 μCi, varying final concentrations) in 100 μl of PKM buffer, pH 6.5, at 20 °C for 5 min. For each concentration of AcCoA, unlabeled coenzyme A-SH was present at the concentrations indicated. The reactions were quenched with 3.5 ml of ice-cold PKM buffer and centrifuged at 100,000 x g for 30 min. The pellets were surface-washed with ice-cold PKM buffer and analyzed for acid-soluble radioactivity as described in the legend to Fig. 1. The results obtained with the acid-insoluble materials are shown as Michaelis-Menten (1/V versus 1/S) plots. The K_i (26.3 μM) value was derived from the negative reciprocal of the point of intersection of the lines. The patterns obtained with the acid-soluble radioactivity and the total radioactivity were similar and are not shown (see text for details).

The acetylation of sialic acids in Golgi vesicles is shown to be an acetylated histidine residue, we questioned if a similar molecule was also the intermediate responsible for donating acetyl groups to the endogenous sialic acids. Prior work by others has shown that DEP can react with both unsubstituted and singly acetylated histidine residues (17). Thus, DEP would prevent the first reaction presumably by reacting with an essential histidine residue. However, if the acetyl intermediate has been preformed, it could still react with the acetylated histidine residue, thereby disabling the second transfer reaction. We therefore prelabeled the intermediate, permeabilized the vesicles, treated with DEP, and looked at the effect upon the second transfer reaction. As shown in Fig. 6, the second transfer reaction was also completely blocked by DEP, indicating the involvement of histidine residue(s) on both sides of the membrane. These experiments of course cannot determine if the histidine residues involved on both sides of the membrane are one and the same.

**Further Evidence for an Essential Lysine—**The lysine-modifying reagent succinic anhydride also blocked the overall reaction (Table II). However, the effect was somewhat variable and dependent upon temperature and pH, presumably because of the instability of this inhibitor in aqueous solution. We therefore examined the effects of another lysine-modifying reagent, TNBS on the reactions (18). Significant inhibition of the overall reaction (>95%) was obtained using 2 mM TNBS (data not shown). Again, the acid-soluble and acid-insoluble components paralleled one another when partial inhibition was achieved at lower concentrations of the reagent. However, TNBS did not have a comparable effect upon the second transfer reaction (44% inhibition at the highest concentration of 2 mM, data not shown). Thus, it appears that the essential lysine residue is important in the first component of the overall reaction. We therefore considered the possibility that the lysine residue is involved in the initial recognition of the anionic phosphomonoester group of the donor acetyl-CoA. In support of this hypothesis, we found that diphospho-CoA-SH was a poorer inhibitor than CoA-SH (only 50% inhibition at 0.1 mM, detailed data not shown). However, because of the difficulty in carrying out protection on the vesicles, the inhibition data for CoA-SH and diphospho-CoA-SH were not directly comparable.

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**Evidence for a Histidine at the Active Site—**These data suggested the possibility of a transmembrane acetylation reaction involving a membrane acetyl intermediate. We therefore considered whether the reaction mechanism might be similar to that described by Rome and his colleagues for the acetylation of glucosamine by lysosomes (14, 15). In that system, acetyl groups donated by acetyl-CoA are transported across the lysosomal membrane by an acetyltransferase with a histidine active site. To investigate this possibility, we studied the effects of various amino acid modifying reagents upon the Golgi acetylation reaction. As shown in Table II, we found that the acetylation of sialic acids in Golgi vesicles is inhibited by NBS and DEP, suggesting the presence of an active site histidine. Unlike the case with the lysosomal reaction, the lysine-modifying reagent succinic anhydride was also effective in blocking the reaction.

Shown in Fig. 5 is a DEP concentration curve, demonstrating substantial inhibition by concentrations of the reagent as low as 0.1–1 μM. As seen in the same figure, we were unable to demonstrate protection by AcCoA present during the DEP reaction at a concentration of 20 μM (7 × apparent K_m). However, higher concentrations of AcCoA could not be tested for two technical reasons. Firstly, the vesicles could not be isolated in an active form after recentrifugation, requiring that simple dilution of the unlabeled AcCoA be used prior to adding the [acetil-\(^{3}H\)AcCoA (the signal is substantially lower) from the unlabeled nucleotide). Secondly, because of ongoing breakdown of acetyl-CoA (11), significant inhibitory quantities of CoA-SH are generated during the protection study, which also cannot be subsequently removed prior to analysis.

If the acetyl intermediate on the external face of the vesicle intermediate) showed no significant inhibition by CoA-SH, even at much higher concentrations (up to 5 mM, data not shown). Acetate at relatively high concentrations (>50 mM) showed partial inhibition of the second reaction, while having no effect upon the overall reaction (see pH curves in Fig. 3 for an example of this effect). Thus, several features indicate that there are two reactions comprising distinct steps in the acetylation mechanism.

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**Further Evidence for an Essential Lysine—**The lysine-modifying reagent succinic anhydride also blocked the overall reaction (Table II). However, the effect was somewhat variable and dependent upon temperature and pH, presumably because of the instability of this inhibitor in aqueous solution. We therefore examined the effects of another lysine-modifying reagent, TNBS on the reactions (18). Significant inhibition of the overall reaction (>95%) was obtained using 2 mM TNBS (data not shown). Again, the acid-soluble and acid-insoluble components paralleled one another when partial inhibition was achieved at lower concentrations of the reagent. However, TNBS did not have a comparable effect upon the second transfer reaction (44% inhibition at the highest concentration of 2 mM, data not shown). Thus, it appears that the essential lysine residue is important in the first component of the overall reaction. We therefore considered the possibility that the lysine residue is involved in the initial recognition of the anionic phosphomonoester group of the donor acetyl-CoA. In support of this hypothesis, we found that diphospho-CoA-SH was a poorer inhibitor than CoA-SH (only 50% inhibition at 0.1 mM, detailed data not shown). However, because of the difficulty in carrying out protection
transmembrane 0-acetyltransferase that can donate acetyl sugar nucleotides for use by luminally oriented transferases. The reaction mixtures were quenched with 4 ml of ice-cold PKM buffer, the vesicles were reisolated, and the acid-soluble and -insoluble radioactivity was determined as described under "Experimental Procedures." The preparation and use of the individual compounds is also described under "Experimental Procedures." All samples were studied in duplicate, and the values are reported as a percentage of the pellet-associated radioactivity found in the absence of inhibitors (4679 cpm of acid-soluble and 4885 cpm of acid-insoluble radioactivity).

### DISCUSSION

The results in this paper and in the preceding one (12) indicate that the O-acetylation of sialic acids has a different mechanism from that of previously described Golgi glycosylation reactions, wherein specific transporters concentrate sugar nucleotides for use by luminally oriented transferases. Taken together, the data indicate that O-acetylation of sialic acids in the rat liver Golgi apparatus may be carried out by a transmembrane O-acetyltransferase that can donate acetyl groups from acetyl-CoA in the cytosol to glycosidically bound sialic acids on luminally oriented endogenous glycoprotein acceptors. If the vesicles are permeabilized with saponin after the initial labeling, transfer can also be demonstrated from an acetyl intermediate to exogenously added free Neu5Ac. Of course, until such time as we are able to purify the relevant protein(s) to homogeneity and reconstitute them into vesicles of defined composition, we cannot be certain about many of the specifics of the acetylation reaction. For example, it is possible that the essential histidine residue(s) on both sides of the membrane are not one and the same, i.e. a relay system might exist between 2 or more histidine residues.

The $K_m$ for transfer to the endogenous sialic acid acceptors experiments (see above), we could not pursue this matter further.

### TABLE II

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<th>Reagent</th>
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</tbody>
</table>

### FIG. 5. DEP concentration curve and lack of protection by AcCoA. Golgi vesicles (0.2 mg) were preincubated in 50 μl of PKM buffer with various concentrations of DEP in the presence or absence of unlabeled AcCoA at a final concentration of 20 μM. After 10 min at 0 °C, the reactions were diluted to 500 μl, and an equal amount of unlabeled AcCoA was added to each tube that did not previously contain it (final AcCoA concentration). Labeled [3H]AcCoA (0.5 μCi) was then added to each tube. The incorporation of label into the Golgi vesicles was determined after incubation at 20 °C for 10 min, as described in the legend to Fig. 6. The data are presented as a percentage of the values obtained in the incubation without DEP (38,096 cpm in the plus AcCoA reaction and 33,070 in the minus unlabeled AcCoA).

### FIG. 6. Effect of DEP upon the second transfer reaction. The transfer of label from [3H]AcCoA-labeled, permeabilized, and washed Golgi vesicles was studied in the presence or absence of DEP or Neu5Ac at the concentrations indicated. The figure shows the paper chromatography profiles obtained in each case with the ethanol-soluble products.
II. O-Acetylated Sialic Acids

TABLE III

<table>
<thead>
<tr>
<th>Lysosomal acetylation</th>
<th>Golgi acetylation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Donor Substrate</strong></td>
<td><strong>Acetyl coenzyme A</strong></td>
</tr>
<tr>
<td>Glucosamine residues in partially degraded heparan sulfate</td>
<td>Sialic acid residues on glycoproteins</td>
</tr>
<tr>
<td>Apparent $K_m$ for acetyl-CoA</td>
<td>0.55 mM</td>
</tr>
<tr>
<td>Apparent $K_m$ for endogenous acceptor</td>
<td>Unknown</td>
</tr>
<tr>
<td>$K_m$ for exogenous monosaccharide acceptor</td>
<td>0.3 mM</td>
</tr>
<tr>
<td>Type of inhibition by CoA-SH for the first reaction</td>
<td>Noncompetitive</td>
</tr>
<tr>
<td>pH optimum of first half-reaction</td>
<td>&gt;8</td>
</tr>
<tr>
<td>pH optimum of second half-reaction</td>
<td>4-5</td>
</tr>
<tr>
<td>Evidence for active site histidine residue in both half-reactions</td>
<td>Yes</td>
</tr>
<tr>
<td>Evidence for essential lysine residues</td>
<td>Yes</td>
</tr>
<tr>
<td>Temperature stability</td>
<td>Stable at 45 °C</td>
</tr>
</tbody>
</table>

The data regarding lysosomal acetylation are taken from Refs. 10, 14, and 15. The data regarding Golgi acetylation are taken from this study and from Ref. 11.

**Fig. 7.** Pathways in the utilization of acetyl coenzyme A by isolated intact rat liver Golgi vesicles. The various reactions studied and elucidated in this paper and in the preceding one (12) are summarized. Definitive evidence for some of the pathways has been presented in some cases. In other instances, the figure presents a model that best fits all the available data. The thickness of the arrows indicates the relative predominance of each pathway. See text for detailed discussion.

with saponin-permeabilized vesicles or with nonionic detergent-solubilized extracts. It is likely that the amounts of saponin required to permit access of the high molecular weight acceptors inside the vesicle irreversibly alter the polypeptides comprising the acetyltransferase. Alternatively, it is possible that the endogenous acceptors are favored because of their pre-existing disposition within the vesicles. We are currently investigating these possibilities.

The data we have presented here closely parallel those reported by Rome and his colleagues in support of transmembrane N-acetylation of glucosamine in lysosomes (10). The major features of the two systems are compared in Table III. It can be seen that there are several features that make it very unlikely that the two mechanisms are mediated by the same protein. These include the nature of the substrates and kinetics of the reactions. However, since nothing is known about the molecular structure of either acetyltransferase, it remains possible that a critical subunit could be shared by the two systems. In the case of the lysosomal system, the relative stability of the transferase and the lack of significant endogenous acceptors made it possible to study the kinetics of the process in great detail, resulting in the demonstration of a Di Isom Ping Pong Bi Bi mechanism (14). However, in the Golgi system, we have not found a way to eliminate the endogenous acceptor. This fact, coupled with the instability of the transferase to reisolation by centrifugation or to excessive permeabilization of the vesicles, makes it difficult to carry out such detailed kinetic studies in this system. Such studies must therefore await the solubilization, purification, stabilization, and reconstitution of the transfer into vesicles of defined composition.

In the lysosomal system, the marked difference in the pH optima of the two half-reactions (>8 and 4-5) permitted the hypothesis that the proton gradient might be the driving force for the acetylation reaction (14). In this Golgi system, the difference in pH optimum is less striking (approximately 7 for the overall process and approximately 6 for the second reaction). However, such values would be in keeping with the known pH differences between the cytosol and the Golgi apparatus (19). On the other hand, our previous studies indicated that stimulation of the Golgi proton pump by ATP had only a modest effect upon the acetylation reaction, while poisons of the proton pump had minimal effects (11).

Outlined in Fig. 7 are proposed pathways for the utilization of acetyl coenzyme A by isolated intact rat liver Golgi vesicles that are based upon the studies described in this paper and in the preceding one. While some aspects of the model are demonstrated conclusively in these studies, others are partly
hypothetical. An acetylhistidine is depicted as the most likely active site residue, and the essential lysine is shown as participating in the first reaction. There may be one or more transferases, each of which could be comprised of multiple polypeptides. Further studies with the purified protein(s) concerned will be necessary to confirm this model and to define the details of the reaction. There are obviously many other questions that remain to be answered. For example, what is the molecular structure of the acetyltransferase(s)? Are di- and tri- O-acetylated sialic acids synthesized by the same transferase(s)? Is migration along the side chain required for the synthesis of such multiply acetylated molecules? Is there a mutase present that has not been uncovered in our *in vitro* studies? Does the esterase activity that is present in the vesicles play a physiological role in the Golgi, or is it simply an enzyme in transit to another location? What is the transfer of free acetate into the lumen that appears to be concomitant to the acetylation reaction? What is the role of the essential lysine residue? Are similar mechanisms involved in the acetylation of sialic acids on other glycoconjugates, such as gangliosides and O-linked oligosaccharides? These and other questions are the subjects of our ongoing work in this area.

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**REFERENCES**