THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 260, No. 11, Issue of June 10, pp. 6600-6608, 1985
Printed in U.S.A.

The Transport and Utilization of Acetyl Coenzyme A by Rat Liver Golgi Vesicles

O-ACETYLATED SIALIC ACIDS ARE A MAJOR PRODUCT*

(Received for publication, December 3, 1984)

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When intact rat liver Golgi vesicles were incubated with [*acetyl*-^{3}H]acetyl coenzyme A, radioactivity was incorporated into the vesicles in a manner dependent upon temperature, time, protein, and acetyl-CoA concentration. The vesicles concentrated the label 121-fold relative to the medium within 20 min, suggesting an active transport mechanism operating in intact vesicles, and incorporated more than 50% of this label into acid-insoluble materials. This was supported by the finding that incorporation was markedly reduced by Triton X-100 at levels above its critical micellar concentration. While the intravesicular low molecular weight fraction was predominantly free acetate, acetate ions themselves were not permeant to the vesicles. Double-label experiments suggested that the transport process involved the entire acetyl-CoA molecule. This was further supported by the fact that coenzyme ASH, palmitoyl-CoA and butyryl-CoA were markedly inhibitory. Incorporation was optimal at 22 °C at pH 7.0, and was moderately stimulated by ATP. However, compounds known to abolish proton gradients or to inhibit the Golgi proton pump had no effect. The apparent K_m for the utilization process was 0.61 μM with a V_{max} of 21.3 pmol/mg of protein/min. Oligomycin and 4,4′-diisothiocyanostilbene-2,2′disulfonic acid were inhibitory, whereas CMP-NeuAc, UDP-GlcNAc, adenosine 3′-phosphate, 5′-phosphosulfate, atractylosides, tunicamycin, 2′5′-ADP, and 3′,5′-ADP were not, showing that this transport process is distinct from other nucleotide transporters previously described in rat liver Golgi. 75–85% of the radioactivity incorporated was shown to be in *O*-acetylated sialic acids, by neuraminidase release, purification, and high pressure liquid chromatography. The majority of the neuraminidase-resistant radioactivity was released by alkaline hydroxylamine as [^{3}H]acetylhydroxamate, but a significant fraction was resistant to this treatment. The nature of the non-sialic acid radioactivity remains unknown. The existence of this transport mechanism provides yet another level at which the *O*-acetylation of sialic acids could be regulated.

The sialic acids are a family of *N*- and *O*-substituted derivatives of neuraminic acid (1). The elegant studies of Blix, Klenk, Gottschalk, Schauer, and others have resulted in the identification of more than 30 different free and glycosidically bound sialic acids in nature (1–3). The most commonly found sialic acid is *N*-acetylneuraminic acid (Neu5Ac).[1] The other sialic acids are all believed to be derived from this molecule by different modifications and substitutions. The most common type of modification is the substitution of *O*-acetyl esters at the hydroxyl groups of the 4-, 7-, 8-, or 9-positions (2, 3). These substitutions are known to have effects upon the action of several enzymes (4–10), upon alternate pathway complement activation (11), and upon bacterial antigenicity and pathogenicity (12).

Earlier studies of the *O*-acetylation reaction were carried out in surviving slices of bovine submaxillary glands, using [^{14}C]acetate as a precursor. These studies, along with the assay of an *O*-acetyltransferase activity in crude homogenates and fractions, were interpreted to indicate that the *O*-acetylation reaction could take place either upon bound or free sialic acids (13–15). We have recently studied the *O*-acetylation reaction by pulse-chase experiments in tissue-culture cell lines, and found that most, if not all, of the *O*-acetylation reaction takes place after the transfer of newly synthesized sialic acids to glycoconjugates.[2] In either model, it is necessary to explain how the donor acetyl coenzyme A which is synthesized in the cytosol gains access to the lumen of the Golgi apparatus, where the sialylation reaction takes place. In the case of the sugar nucleotides, Hirschberg, Sandhoff, and others (16–23) have clearly shown evidence for the existence of specific transporter proteins that mediate the entry of such molecules into the Golgi apparatus. We postulated the existence of a similar carrier mechanism for acetyl-CoA. In this study, we demonstrate and characterize such an activity in isolated rat liver Golgi vesicles, and show that *O*-acetylated sialic acids are the major (but not the sole) products. The transport activity appears to be distinct from the others previously described in the Golgi, and from that described by Rome *et al.* (24) in intact isolated lysosomes.

EXPERIMENTAL PROCEDURES

Materials—Most of the biological reagents used here were obtained from Sigma. The following materials were obtained from the sources

* This work was supported by United States Public Health Service Grant GM32373 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The various sialic acids are designed by combinations of Neu (neuraminic acid), Ac (acetyl), and Gc (glycolyl). The amino group at the 5-position is always substituted with an acetyl (Ac) or a glycolyl (Gc) group. Other substitution positions are indicated by numerals. For example, *N*-glycolylneuraminic acid is written as Neu5Gc and *N*-acetyl-9-mono-*O*-acetylneuraminic acid as Neu5,9Ac$_2$ (after Schauer and others (2)). Other abbreviations used are: HPLC, high pressure liquid chromatography; PAPS, adenosine 3′-phosphate,5′-phosphosulfate.

[2] A. Varki and S. Diaz, unpublished observations.

indicated: Dowex 50 AG 1-X2 (H^+ form) and Dowex 3-X4A (100–200 mesh, chloride form), Bio-Rad; chemically synthesized Neu5Ac (>99% purity), Kantoishi Pharmaceutical Co., Tokyo, Japan; *Vibrio cholerae* and *Arthrobacter ureafaciens* neuraminidases, Calbiochem-Behring; [4-^{14}C]*N*-acetylneuraminic acid (56.8 mCi/mmol), [1-^{14}C] acetyl coenzyme A (53.5 Ci/mmol), [G-^{3}H]coenzyme A (2.8 Ci/mmol), [*sialic*-9-^{3}H]CMP-sialic acid (18.9 Ci/mmol), and [*sialic*-4,5,6,7,8,9-^{14}C]CMP-sialic acid (247 mCi/mmol), New England Nuclear; [^{3}H] methoxyinulin (175 mCi/mg), [1,2-^{3}H]deoxy-D-glucose (28 Ci/mmol), and [*acetyl*-^{3}H]acetyl coenzyme A (1.3 or 3.4 Ci/mmol), ICN Pharmaceuticals Inc.; [1-^{14}C]acetate (57 mCi/mmol) and UDP-[6-^{3}H] galactose (15.6 Ci/mmol), Amersham Corp.

Neuraminidase from *Streptococcus sanguis* was prepared as previously described (6). Authentic Neu5,9Ac_2 was kindly provided by Professor Roland Schauer, Christian Universitat, Kiel, Federal Republic of Germany. The Dowex 3-X4A resin was converted to the formate form, as recommended by the manufacturer. All other chemicals were of reagent grade and were purchased from commercial sources.

Isolation and Characterization of Rat Liver Golgi—Intact rat liver Golgi vesicles were prepared by the method of Leelevathi (25). The intactness and sidedness of Golgi vesicles were determined exactly as described by Carey and Hirschberg (16). Galactosyltransferase (26–28), glucose-6-phosphatase (29), and β-galactosidase (30) were measured as previously described. Protein was measured by the method of Lowry *et al.* (31).

The utilization of acetyl-CoA by Golgi vesicles was assayed by incubating 25–500 μg of vesicle protein in 0.5–1 ml of TKM (10 mM Tris-HCl, 150 mM KCl, 1 mM $MgCl_2$, pH 7.5) or PKM (10 mM potassium phosphate, 150 mM KCl, 1 mM $MgCl_2$, pH 7.0) buffer at 22 °C in the presence of [^{14}C]- or [^{3}H]acetyl-CoA at various concentrations, as indicated. For some studies the reactions were quenched with 4 ml of ice-cold buffer, the vesicles were re-isolated by centrifugation, washed, and sonicated, and the perchloric acid-soluble and -insoluble radioactivity was determined exactly as described by Carey and Hirschberg (16). Some of the studies were performed by directly quenching the reactions with an equal volume of ice-cold 8% perchloric acid and assaying only the acid-precipitable radioactivity. These samples were incubated on ice for 15 min and spun at 10,000 × *g* for 10 min, the supernatant was removed, and the pellet was surface-washed once with 4% perchloric acid. The pellet was then resuspended in 1 ml of 4% perchloric acid, re-isolated by centrifugation, and dissolved in 700 μl of 1 N NaOH by heating at 55 °C for 15 min. The dissolved material was then neutralized with 350 μl of 2 N HCl, and 1 ml of the mixture was counted with 10 ml of Aquasol.

Chromatographic Methods—Acylhydroxamates were prepared by alkaline hydroxylamine treatment, and separated by thin layer chromatography as described by Schauer (32). The chromatograms were dried and cut into 0.5-cm strips, which were soaked in 0.5 ml of water and 5 ml of Aquasol for determination of radioactivity. The radioactive compounds were identified by comparison with an internal [^{14}C] acetylhydroxamate and other external nonradioactive hydroxamate standards, which were prepared from the appropriate esters (32). HPLC was used to separate acetyl coenzyme A, coenzyme A, and free acetate by a modification of a previously described reversed-phase ion-pair method (33). An Alltech RP-18 column (250 × 4.1 cm) was eluted in the isocratic mode with 38% methanol, 0.5% tetrabutylammonium formate, pH 4.5, at 1 ml/min (System A). Under these conditions, free acetate, CoA, and AcCoA elute at 2, 10, and 15 min, respectively (not shown). The elution of unknown ^{3}H-compounds was compared with that of external standards (monitored at A_{254}) and internal [^{14}C]acetate and [^{14}C]acetyl-CoA standards. Various types of sialic acids were separated from each other by HPLC on a Varian Micropak AX-5 column eluted in the isocratic mode with acetonitrile:water:0.5 M NaH_2PO_4 (64:26:10) (System B). This method is based on the ion suppression:amine absorption principle used for separation of acidic oligosaccharides by Mellis and Baenziger (34). As shown in Fig. 1, it is capable of separating a variety of mono- and di-*O*-acetylated derivatives of *N*-acetylneuraminic and *N*-glycolylneuraminic acid from each other. The elution of unknown radioactive compounds was monitored in comparison to the nonradioactive standards (detected by absorption at 200 nm) and to radioactive standards obtained from metabolically labeled murine erythroleukemia and myeloma cells.[2] In some cases, the sample was eluted at 1 ml/min with a linear gradient change to 54% acetonitrile, 36% water, 10% phosphate buffer over 20 min. The gradient gives somewhat earlier elution times, but sharper resolution.

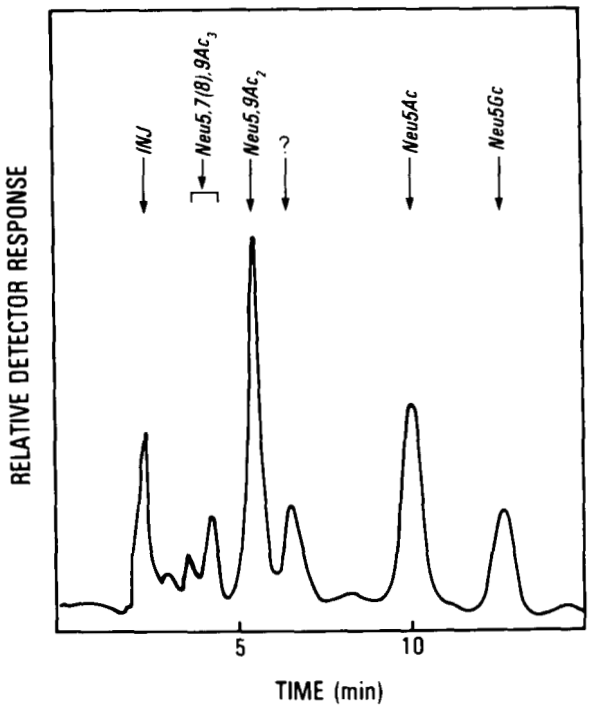

FIG. 1. **HPLC for the separation of various sialic acids.** Work was carried out with a Spectra-Physics 8700 Ternary Gradient elution system on a Varian Micropak AX-5 column (30 × 0.4 cm). The solvents used were acetonitrile (A), water (B), and 0.5 M sodium dihydrogen phosphate, pH 4.3 (C). The column was equilibrated in 64% A, 26% B, 10% C and eluted in the isocratic mode; the sample was loaded into a 50-μl loop. An example of such a separation is shown here, using a mixture of sialic acids purified from bovine submaxillary mucin; the elution was monitored at 200 nm, with the chart speed at 1 cm/min. The position of known standards is as indicated. *INJ*, injection peak.

Further Fractionation of Labeled Golgi Vesicles—The vesicles were washed in the same buffer used for the labeling and then sonicated in 1 ml of 10 mM pyridinium formate buffer, pH 5.5, using 4 × 15-sec pulses of a Heat Systems-Ultrasonics sonicator cell disruptor model W-185-F with a probe setting of 30. The sonicate was centrifuged at 100,000 × *g* for 30 min; the resulting pellet was called the "membrane" fraction. It was washed once in 10 mM pyridinium formate, pH 5.5, and resuspended in 1 ml of the same buffer. The 100,000 × *g* supernatant was adjusted to 90% absolute ice-cold ethanol and placed at −20 °C for 1 h. The flocculent precipitate was collected by centrifugation at 1,500 × *g* for 15 min and then resuspended in 1 ml of 5 mM pyridinium formate, pH 5.5. The ethanol supernatant was evaporated under reduced pressure and brought up in 1 ml of 10 mM pyridinium formate, pH 5.5. All fractions were monitored for radioactivity and stored at −20 °C until future analysis.

Release, purification, and analytical de-*O*-acetylation of sialic acids were carried out exactly as previously described (35) except for the following changes: The addition of formic acid to the Dowex-50 eluate was omitted; if the pH of the pooled Dowex-50 eluate was greater than 3, it was applied directly to the Dowex 3-X4A column.

RESULTS

Characterization of Intactness and Purity of Golgi Vesicles—Rat liver Golgi vesicles were isolated and characterized as described under "Experimental Procedures." A typical preparation (used for many of the studies reported here) was enriched 103-fold for galactosyltransferase (61% yield), 2.3-fold for glucose-6-phosphatase (1.4% yield), and 2.2-fold for β-galactosidase (1.3% yield). The intactness of the vesicles was judged by their latency toward neuraminidase-catalyzed removal of labeled sialic acid exactly as described by Carey and Hirschberg (16). By this criterion, greater than 90% of

the vesicles were sealed and of the correct topographic orientation, in all Golgi preparations used in this study.

The Incorporation of Radioactivity from [^{3}H]Acetyl-CoA into Rat Liver Golgi Vesicles—The intact, right-sided liver Golgi vesicles were incubated with [^{3}H]acetyl-CoA in TKM buffer as described under "Experimental Procedures." As shown in Fig. 2, these vesicles incorporated acid-soluble and acid-insoluble radioactivity from [^{3}H]acetyl-CoA in a manner dependent upon time, temperature, protein concentration, and acetyl-CoA concentration. The reaction was not linear with time beyond 5 min (detailed data not shown). The incorporation at 0 °C (which could include any occurring during the subsequent 4 °C centrifugation step) ranged from 20 to 40% of that at room temperature in various experiments. Under all conditions studied, the acid-soluble radioactivity closely paralleled the acid insoluble component and represented from 25 to 40% of the pellet-associated radioactivity. The incorporation of radioactivity seemed to approach saturation with the addition of increasing amounts of radioactive acetyl-CoA in the 3–5 μM range. Addition of an excess of nonradioactive acetyl-CoA (at 40 μM) markedly reduced the incorporation of radioactivity (see Fig. 2). These findings suggested the existence of a specific mechanism for the utilization of acetyl-CoA by the Golgi vesicles.

Evidence for Accumulation and Concentration of Radioactive Compounds within the Vesicles—If an active transport process were involved in these phenomena, the radioactivity should show a relative concentration in the vesicles over that in the medium. Hirschberg and others (17, 20) have previously shown that impermeant ([^{3}H]methoxyinulin) and permeant ([1,2-^{3}H]deoxyglucose) markers can be used to accurately estimate the mean total pellet volume, [V_t], of Golgi vesicles, the volume outside the pellet, [V_o], and thus the internal volume of the pellet, [V_i] (= [V_t] − [V_o]). This information was compared with that of the incorporation of various radioactively labeled nucleotide sugars under identical conditions to obtain a "penetration index" in each case. This then allowed a determination of the fold concentration of the label in the vesicles achieved by the transport system in question. We used the identical approach to ask if the Golgi vesicles were indeed concentrating the label from [^{3}H]acetyl-CoA. The details of the experiment are described in Table I. Briefly, when Golgi vesicles were incubated in TKM buffer for 20 min at 22 °C with [^{3}H]acetyl-CoA at 1.54 μM, the total of radioactive solutes in the pellet was 86.9 pmol/mg of the radioactivity. Based upon the [V_i] value (0.26 μl/pellet volume/mg of Golgi protein) obtained under identical conditions, the concentration of radioactivity inside the pellet was calculated to be 187.1 μM. This represents a 121-fold concentration of the label within the vesicles over that in the medium. This is very similar to the findings of Hirschberg and others (17, 20) for other nucleotides such as CMP-sialic acid, GDP-fucose, and PAPS and suggests that a similar active transport mechanism is present for acetyl-CoA in Golgi vesicles.

Characterization of Intravesicular Low Molecular Weight Contents—As described above, the intravesicular low molecular weight (acid-soluble) radioactivity represented from 25 to 40% of the total radioactivity incorporated and closely correlated with the acid-insoluble fraction under various conditions. We analyzed this fraction further by HPLC in System A as described under "Experimental Procedures." We found that greater than 99% of the label was in the form of free acetate. We next studied the radioactivity remaining in the medium after a 20-min incubation with 1 mg of Golgi vesicles in TKM buffer. We found that 60% of this label was now also in the form of free acetate. An identical incubation in the absence of Golgi vesicles resulted in only 4% breakdown of the acetyl-CoA. This rapid breakdown of the added acetyl-CoA was thus being catalyzed by the presence of the Golgi vesicles. This also raised the possibility that the incorporation of label observed was actually that of free acetate rather than the acetyl-CoA molecule.

Free Acetate Is Not Taken up by Golgi Vesicles—To investigate this possibility we incubated Golgi vesicles with a

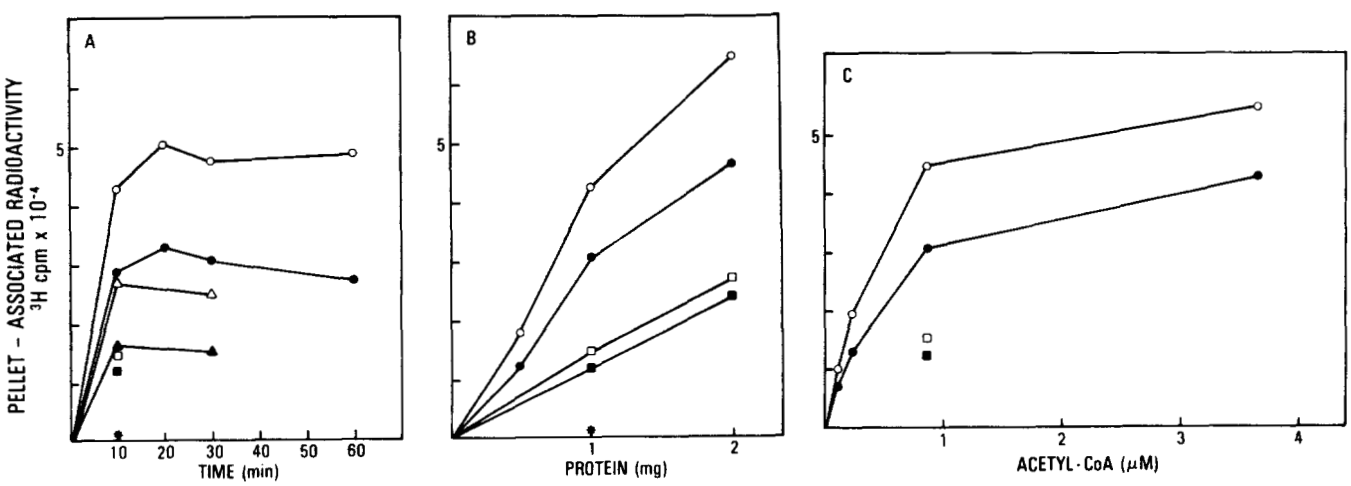

FIG. 2. **Incorporation of radioactivity into isolated rat liver Golgi vesicles from [*acetyl*-^{3}H]acetyl-CoA.** Golgi vesicles were preincubated in TKM for 30 s, in the presence or absence of ATP (0.5 mM final concentration). The reaction was started by the addition of varying amounts of [^{3}H]acetyl-CoA, and the incubation was conducted under varying conditions of temperature, time, and protein concentration. The reaction mixtures were then centrifuged at 100,000 × *g* for 30 min at 4 °C, and the pellet-associated radioactivity (acid-soluble and acid-insoluble) was determined as described under "Experimental Procedures." The various points show incubations under the following conditions: 22 °C, +ATP (○, ●); 22 °C, −ATP (▲, △); 0 °C, +ATP (□, ■); and 22 °C, +ATP, +40 μM unlabeled acetyl-CoA (*). The open symbols represent acid-insoluble radioactivity, and the closed symbols represent acid-soluble radioactivity in each case. The *asterisk* represents total pellet-associated radioactivity. The studies in *panels A* and *C* were carried out with 1 mg of Golgi protein; those in *panels A* and *B* were carried out with [^{3}H]acetyl-CoA at 0.9 μM; those in *panels B* and *C* were carried out for 10 min at the temperatures indicated.

TABLE I

Evidence for translocation of [^{3}H]acetyl-CoA into Golgi vesicles: concentration of radioactive solutes within the vesicles

Golgi vesicles (0.25 mg of protein) were incubated with [*acetyl*-^{3}H] acetyl-CoA (1.54 nmol, 4.4 × 10^6 dpm) at 22 °C for 10 min in a final volume of 1 ml of TKM buffer, containing 0.5 mM ATP. Parallel identical incubations were made with [^{3}H]inulin and [^{3}H]deoxyglucose. The vesicles were then chilled and re-isolated, and the radioactive solutes were determined as described under "Experimental Procedures." Mean values of duplicate determinations were used to calculate the following parameters *exactly* as described elsewhere by Hirschberg and others (17, 20). [S_m] is the concentraton of the acetyl-CoA in the media; [V_t] is the total volume of the pellet determined from the deoxyglucose control; [V_o] is the volume trapped outside the pellet, and is determined from the inulin control; [V_i] is the volume inside the pellet determined by subtracting the two former values; [S_t] is the total radioactive solute in the entire pellet; [S_o] is the solute outside the vesicles; and [S_i] is the solute present inside the vesicles.

[S_m]	Radioactive solutes in pellet			Volume of Golgi pellet			[S_i]	$\frac{[S_i]}{[S_m]}$
	S_t	S_o	S_i	V_t	V_o	V_i		
μM	*pmol/mg protein*			*μl/mg protein*				
1.54	86.8	38.2	48.6	0.70	0.44	0.26	186.9	121.4

TABLE II

Uptake and incorporation of labeled molecules into Golgi vesicles

Rat liver Golgi vesicles (1 mg of protein) were incubated at 22 °C in TKM buffer with 0.5 mM ATP and the radiolabeled compounds at the concentrations indicated. After 20 min, 3 ml of ice-cold TKM buffer was added, the vesicles were reisolated, and radioactivity was determined.

Radioactive compounds added	Final concentration	Radioactivity found in:		
		Media (starting)	Pellet-associated	
			Acid-soluble	Acid-insoluble
	μM		*dpm*	
[^{3}H]Acetyl-CoA	1.0	3,052,398	124,111	196,628
[1-^{14}C]Acetate	5.0	696,302	561	89
(^{3}H/^{14}C Ratio)		(4.4)	(221)	(2209)
[^{14}C]Acetyl-CoA	3.0	1,061,040	46,985	73,950
Acetyl-[^{3}H]CoA	0.2	414,960	11,387	8,584
(^{3}H/^{14}C Ratio)		(0.391)	(0.242)	(0.116)
[^{3}H]Coenzyme ASH	0.2	1,283,632	106,638	145,458
(% media dpm)		(100)	(8.3)	(11.3)
[^{3}H]Deoxyglucose	0.15	11,676,578	8,260	766
(% media dpm)		(100)	(0.07)	(0.006)

mixture of [^{14}C]acetate and [^{3}H]acetyl-CoA. As shown in Table II, in spite of a 5-fold molar excess of the free acetate, the ^{14}C label was taken up at less than 1% of the rate seen for the ^{3}H label. This shows that although free acetate was found both outside and inside the vesicles at the end of the incubation, the intact acetyl-CoA molecule was involved in the incorporation seen. This also shows that significant conversion of free acetate to acetyl-CoA is not taking place in the presence of the added ATP.

Evidence for Uptake of the Entire Acetyl Coenzyme A Molecule—To confirm that the entire acetyl-CoA molecule was being taken up, we compared the uptake of simultaneously added [^{14}C]acetyl-CoA with acetyl-[G-^{3}H]CoA. The acetyl-[G-^{3}H]CoA was prepared by acetylation of [G-^{3}H]CoASH by treatment with acetic anhydride, as described by Stadtman (36). As shown in Table II, we found that the ^{3}H/^{14}C ratio of the intravesicular acid-soluble fraction was very close to that of the starting media, strongly suggesting that both the acetyl and the CoA portions of the molecule were being transported. Surprisingly, we found that ^{3}H radioactivity from the [G-^{3}H] coenzyme A portion of the molecule was also being incorporated into acid-insoluble materials at a significant rate. As a "control" we had also incubated the Golgi vesicles with [^{3}H] CoA. In this case, we found that there was again a considerable concentration of the label in the vesicles relative to the [^{3}H] deoxyglucose control marker (see Table II), suggesting vectorial transport of coenzyme ASH. We are uncertain of the nature of the radioactivity incorporated from the [^{3}H]CoA into acid-insoluble materials. Since this labeled compound was originally prepared by ^{3}H gas exchange into all available sites, much further work must be done to confirm the significance of this finding.

Nonionic Detergents Abolish Uptake and Utilization—As shown in detail below, [^{3}H]*O*-acetylsialic acids were found to be a major component of the acid-insoluble fraction. These products are presumably formed by the action of *O*-acetyltransferases on endogenous acceptors, utilizing the [^{3}H]acetyl-CoA as a donor. Thus, the incorporation of radioactivity into the acid-insoluble materials could be limited by at least two factors: the rate of transport of the donor molecule into the vesicles or the activity of the *O*-acetyltransferase(s). To differentiate between these two possibilities, we investigated the effects of the nonionic detergent Triton X-100 on the incorporation of radioactivity into acid-insoluble materials. As demonstrated in Fig. 3, we found that the detergent was without effect until the concentration approached that of its critical micellar concentration (0.015%, see Ref. 37). Above this level, where the acetyl-CoA should be freely accessible to the *O*-acetyltransferases inside the lumen, the incorporation was markedly diminished. As a positive control, we showed (see Fig. 3) that the activity of the Golgi enzyme galactosyltransferase toward an *exogenous* acceptor (free GlcNAc at 100 mM) was greatly enhanced above the critical micellar concentration of the detergent. The most likely explanation for these findings is that at the concentration of acetyl-CoA used (1.3 μM) the rate-limiting step was the transport process rather

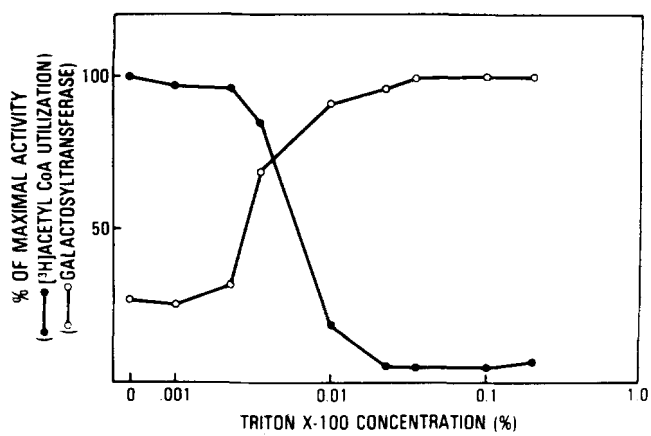

FIG. 3. **Effects of nonionic detergent on Golgi utilization of [^{3}H]acetyl-CoA and galactosyltransferase activity.** For [^{3}H] acetyl-CoA utilization, Golgi vesicles were preincubated in TKM buffer with Triton X-100 at the concentrations indicated for 30 s. The label (0.9 μM final concentration) was added, the reaction was quenched after 6 min with perchloric acid, and the acid-insoluble radioactivity was determined as described under "Experimental Procedures." For determination of the activity of galactosyltransferase toward the *exogenous* acceptor GlcNAc, the reaction was started by addition of the enzyme to a tube containing all other elements of the assay. The transfer of label from UDP-[6-^{3}H]galactose to GlcNAc was determined exactly as described elsewhere (27), under conditions that were linear with time and protein concentration.

than the *O*-acetyltransferase activities. To rule out the possibility that the detergent was inactivating the enzyme(s) above its critical micellar concentration, we also studied the effects of increasing the acetyl-CoA concentration. As shown in Fig. 4, the incorporation of acetate into acid-insoluble material was saturable with acetyl-CoA in the 5–10 μM range with intact Golgi vesicles. In the presence of 0.2% Triton X-100, significant activity did eventually appear in the 10–100 μM range. This suggests that the *O*-acetyltransferases were still intact, but could show significant activity only at much higher concentrations of the donor molecule. This is in keeping with previous studies of an *O*-acetyltransferase activity in submaxillary glands, which had a K_m for acetyl-CoA of 100 μM (15). The only other relatively unlikely explanation of these findings is that the detergent could be modifying the K_m of the enzyme itself.

Optimization of Conditions for Further Studies—Further studies of the kinetics and requirements of the reaction were greatly hampered by the very significant activity seen at 0 °C (see above), which presumably also represented uptake occurring during the subsequent 4 °C centrifugation step. Not surprisingly, this "background" activity was highly variable, depending upon the exact handling conditions of each sample. This problem was further compounded by our finding (see below) that the Golgi vesicles contain an endogenous esterase activity. These facts, coupled with the lack of linearity of the assay beyond very short time points, made it very difficult to obtain valid blanks and accurate time points using the centrifugation method. We therefore carried out all further studies of the reaction by directly quenching the reactions with perchloric acid and directly determining the acid-precipitable radioactivity. This eliminated analysis of the acid-soluble radioactivity within the vesicles. However, as indicated above, in all cases studied by centrifugation, the radioactivity in the acid-soluble fraction closely paralleled that in the insoluble fraction. Furthermore, the detergent experiments described above strongly suggest that the intactness of the vesicles (and therefore the transport process) is the rate-limiting factor in the incorporation of radioactivity into acid-insoluble materials. Thus, the utilization of radioactivity by the vesicles under various conditions is probably an indirect measure of the transport process itself.

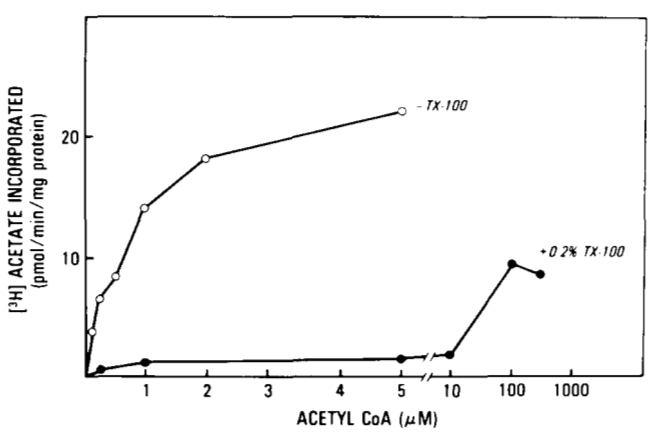

FIG. 4. **Effects of increasing acetyl-CoA concentration in the presence and absence of detergent.** Golgi vesicles were preincubated for 30 s in TKM buffer in the presence or absence of 0.2% Triton X-100 (final concentration). The reaction was started by addition of [³H]acetyl-CoA at the various concentrations indicated. The reactions were quenched after 6 min with perchloric acid, and the acid-insoluble radioactivity was determined as described under "Experimental Procedures." The incorporation of acetate was calculated based upon the specific activity of the added label in each case.

Using this approach, we studied the effects of various buffers, salts, divalent cations, pH values, and other agents on the process during 3–5-min incubations. Maximal activity was seen at pH 7.0 in the presence of potassium phosphate buffer (20 mM) and potassium chloride (150 mM). Other attempts to manipulate the buffer composition to more closely mimic the normal composition of natural cytosolic salts (38) (*viz.* addition of sulfate, increase of phosphate with lowering of chloride) did not improve the activity. Under the optimal buffer conditions and in the presence of ATP, the linearity of the assay was greatly improved (completely linear up to 8 min, data not shown). Using these optimal salt and buffer concentrations, the effects of other agents were studied. The addition of 2-mercaptoethanol (a reducing agent), 2,3-dimercaptopropanol (a reducing agent and zinc chelator which inhibits pyrophosphatases) (39), sodium fluoride (an inhibitor of pyrophosphatases) (40, 41), or bovine serum albumin (1 mg/ml) removal of divalent cations from the buffer, substitution of Ca^{2+} for Mg^{2+}, or addition of 2 mM EDTA were without remarkable effect on the process.

Effects of Substrate Analogues and Other Compounds—The studies so far suggested that the intact acetyl-CoA molecule was involved in the utilization process. We further explored the specificity of this reaction by studying the effects of various substrate analogues and other compounds known to be taken up by rat liver Golgi vesicles. As shown in Table III, the utilization process was markedly inhibited by coenzyme ASH, butyryl-CoA, and palmitoyl-CoA in concentrations in the 1–5 μM range. These concentrations are well below the critical micellar concentration of these molecules (42, 43),

TABLE III

Effects of substrate analogues and other compounds on the incorporation of [³H]acetyl-CoA into rat liver Golgi vesicles

Rat liver Golgi vesicles (215 μg of protein) were preincubated in 500 μl of PKM buffer with 1 mM ATP and the various compounds at the concentrations indicated for 3 min at 22 °C. [³H]Acetyl-CoA (1 μCi) was added, and the reactions were quenched after 6 min with 500 μl of 8% perchloric acid. The acid-precipitable radioactivity was then determined exactly as described in the legend to Table II and expressed as a percentage of that obtained in a control incubation with no additions. Some of the compounds were added as solutions in absolute ethanol. The final concentration of ethanol never exceeded 0.5% in any incubation. This concentration of ethanol had no significant effects on the reactions (not shown). DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid.

Compound	% of control activity at concentration				
	0.1 μM	1 μM	5 μM	25 μM	100 μM
Coenzyme ASH	87	55	31	8	3
Butyryl-CoA	89	73	58		14
Palmitoyl-CoA	90	59	35		3
CMP-Neu5Ac		85	80	90	91
UDP-GlcNAc		85	83	90	77
Atractyloside			74	85	89
Carboxylatractyloside			87	78	91
DIDS			75	54	13
PAPS	87	105	78		
2′,5′-ADP		94	94	81	
3′,5′-ADP		95	90	96	

	% of control activity
Tunicamycin	
(0.1 μg/ml)	91
(1.0 μg/ml)	88
(5.0 μg/ml)	88
Oligomycin (50 μg/ml)	56
Ammonium sulfate (10 mM)	84
Monensin (2 μM)	96
Sodium vanadate (0.1 mM)	89
N-Ethylmaleimide (0.2 mM)	95

implying that the inhibition is not related to a detergent effect. This suggests that the coenzyme A moiety is recognized during the process and supports the findings with the free acetate and double-labeled compounds, described above. Previous studies have indicated the existence of independent transport mechanisms for several other nucleotides in rat liver Golgi vesicles. As shown in Table III, we found that CMP-sialic acid (16, 17) and UDP-GlcNAc (22) were without significant effect upon the acetyl-CoA utilization. Tunicamycin, which inhibits UDP-galactose transport by Golgi vesicles (18, 19), was also without effect. The sulfate donor PAPS is similar to acetyl-CoA in two ways. First, both molecules contain a 3′,5′-ADP group, and second, palmitoyl-CoA has been previously shown to be a good inhibitor of PAPS uptake by rat liver Golgi (21). However, as shown in Table III, the acetyl-CoA utilization process under study here is quite different in other respects from the PAPS system. First, coenzyme A was not found to affect PAPS uptake (21), while 3′,5′-ADP, atractyloside, and carboxyatractyloside, which inhibit PAPS uptake (21), were without effect on the acetyl-CoA uptake. Second, PAPS itself had little effect in concentrations well above its previously reported K_m of 0.7 μM (20). Besides the inhibition by palmitoyl-CoA, the only other similarity between the two processes was the inhibition by the nonspecific anion transport inhibitor 4,4-diisothiocyanostilbene-2,2' disulfonic acid (21). These findings show that the acetyl-CoA uptake process described here is different from all previously described Golgi uptake mechanisms.

Further Investigation of the ATP Effect—With the use of the improved buffer and pH conditions, the ATP effect became less pronounced. A maximum of 28% enhancement was seen at 0.5 mM ATP in PKM buffer at 22 °C. Higher concentrations of ATP had no further effect; in fact, concentrations above 3 mM caused inhibition. The addition of an ATP-regenerating system (creatine phosphate at 1.2 mM and creatine phosphokinase at 0.125 mg (150 units/ml)) also did not improve the ATP effect, further suggesting that ATP breakdown was not the limiting factor in the ATP effect. The uptake at 0 °C, which was 37% of that at 22 °C in this experiment, was unaffected by ATP. Preincubation of the Golgi vesicles in 0.5 mM ATP for 10 min prior to the addition of the acetyl-CoA did not provide any improvement over the effect of simultaneous addition.

The Effects of ATP Are Not Related to the Golgi "Proton Pump"—Recent studies have demonstrated the existence of a Golgi proton pump that utilizes ATP or GTP in the presence of Mg^{2+}, to maintain an acidic pH in the interior of the Golgi vesicles (44, 45). The lack of dependence of the ATP effect on Mg^{2+} already suggested that it was not due to enhancement of the ATPase function. To confirm this, we demonstrated (see Table III) that *N*-ethylmaleimide, which inhibits the Golgi proton pump (45), and agents such as ammonium sulfate and monensin, which would collapse a pH gradient, were without significant effect on the process. Unexpectedly, oligomycin, which affects the mitochondrial Mg^{2+}-ATPase and should not affect the Golgi proton pump (45), was capable of inhibiting the acetyl-CoA utilization process. Sodium vanadate, which affects certain other ATPases, was without effect.

Kinetics of the Uptake Process—Because of the difficulties with obtaining true blanks under various conditions (see above), an accurate study of the kinetics of the uptake process was not possible. Therefore, we studied the kinetics of incorporation of the radioactivity from [^{3}H]acetyl-CoA into acid-insoluble materials alone. As shown in Fig. 5, this gave an apparent K_m of 0.61 μM, with a V_{max} of 21.73 pmol/mg/min. Since our earlier studies had suggested that the rate-limiting

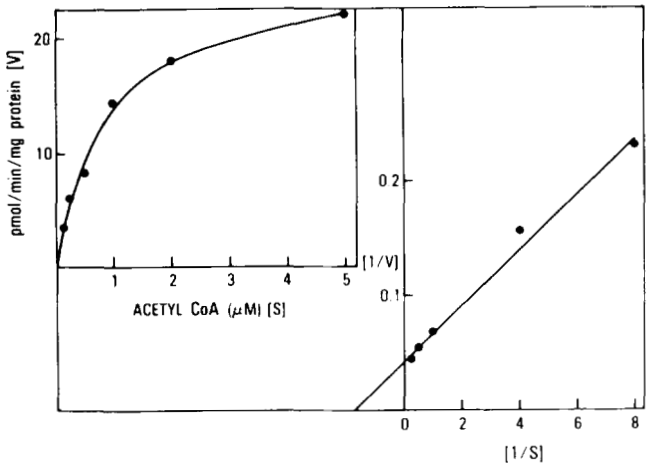

FIG. 5. **Kinetics of incorporation of label from [*acetyl*-^{3}H] acetyl-CoA into rat liver Golgi vesicles.** Golgi vesicles were preincubated for 30 s at 22 °C in PKM buffer. The reactions were started by addition of [^{3}H]acetyl-CoA in various amounts for the final concentrations indicated. The reactions were quenched after 5 min by addition of perchloric acid, and the acid-insoluble radioactivity was determined as described under "Experimental Procedures." All points shown are the mean of duplicate determinations. The data are presented as a function of [*V*] *versus* [*S*] and as a double-reciprocal plot. The latter was used to calculate an apparent K_m of 0.6 μM and a V_{max} of 21.73 pmol/mg/min.

factor in the accumulation of the acid-insoluble radioactivity was probably the transport process itself, the K_m value obtained here is probably a reasonable approximation of the kinetics of the transport process. More accurate studies must await the purification of the transport protein itself.

Stability—The activity was markedly unstable at 37 °C. About 40% of the activity was lost upon preincubation at room temperature for 10 min, regardless of whether ATP was present. A slower but significant loss of activity occurred even at 0 °C. The activity appeared stable when the vesicles were gently suspended into 10 mg/ml bovine serum albumin (with a Dounce homogenizer) and stored in liquid nitrogen for up to 2 weeks. However, prolonged storage under these conditions also resulted in gradual loss of activity. A single cycle of repelleting and resuspension caused complete loss of activity. Thus, it was not possible to carry out some types of useful experiments (*e.g.* pretreatment with Pronase) (17).

Characterization of Macromolecular Products—We next characterized the nature of the products formed within the Golgi vesicles during the incubation with [^{3}H]acetyl-CoA. A single large preparation of Golgi vesicles was made in PKM buffer for 30 min. The labeled vesicles were pelleted, washed, and disrupted by sonication in hypotonic buffer, and the membranes were reisolated by centrifugation, as described under "Experimental Procedures." The labeled membranes thus obtained contained 60% of the total radioactivity originally found in the vesicles. The soluble macromolecular products from the supernatant were precipitated with 90% cold ethanol and contained 10% of the total label, leaving 30% of the total label in the ethanol-soluble fraction. For the following characterization of the "macromolecular" fraction, the membranes and the resolubilized ethanol precipitate were then pooled together.

Our original prediction of the existence of this transport mechanism was based upon the expectation that it would make the donor available for a Golgi-localized sialic acid-specific *O*-acetyltransferase. We therefore first looked for the presence of [^{3}H]-*O*-acetylsialic acids in the membrane-bound fraction. An aliquot of the labeled macromolecular fraction

was treated with a mixture of neuraminidases from *S. sanguis*, *V. cholerae*, and *A. ureafaciens* (35), which would release most known sialic acids. The incubation conditions were as previously described (35), except for the addition of 0.2% Triton X-100, to ensure maximal exposure of all macromolecules to the enzymes. In a parallel control experiment, these conditions were shown to release greater than 90% of the label incorporated into Golgi vesicles from CMP-[^{14}C]NeuNAc. This treatment resulted in release of 75–85% of ^{3}H label from [^{3}H]acetyl-CoA-labeled membranes in different experiments. The released label was further purified using a previously described method (35) which gives an 80–90% yield of sialic acids with less than 5% loss of *O*-acetylation. When an aliquot of the purified label was treated with alkaline hydroxylamine and studied by TLC, almost all of the radioactivity co-migrated with [^{14}C]acetylhydroxamate, and away from lactyl-, glycolyl-, butyryl-, and palmitoylhydroxamates (not shown). This suggests that the incorporated label was primarily in *O*-acetyl esters, and that other acyl groups were not being formed. Another aliquot of the purified label was studied by a HPLC method which separates the different types of sialic acids. As shown in Fig. 6, all of the label was retained by the HPLC column and the major peaks eluted in positions characteristic for mono- and di-*O*-acetylated sialic acids (compare with Fig. 1). As expected, none of the peaks eluted in the position of non-*O*-acetylated Neu5Ac or Neu5Gc. All of the label could be converted into free acetate (see *lower panel* of Fig. 6) by treatment with base under conditions known to de-*O*-acetylate sialic acids. In all, there were at least eight such distinct base-labile peaks. Since all of these compounds were released by neuraminidase and copurified through several steps with sialic acids, it is reasonable to assume that they are all *O*-acetylated sialic acids. They could represent various *O*-acetylated derivatives of endogenous Neu5Ac and Neu5Gc and also of other endogenous sialic acids that were already substituted with other groups (*e.g.* methyl groups) (2). Since the label is exclusively in the *O*-acetyl groups, it is very difficult to characterize these compounds further.

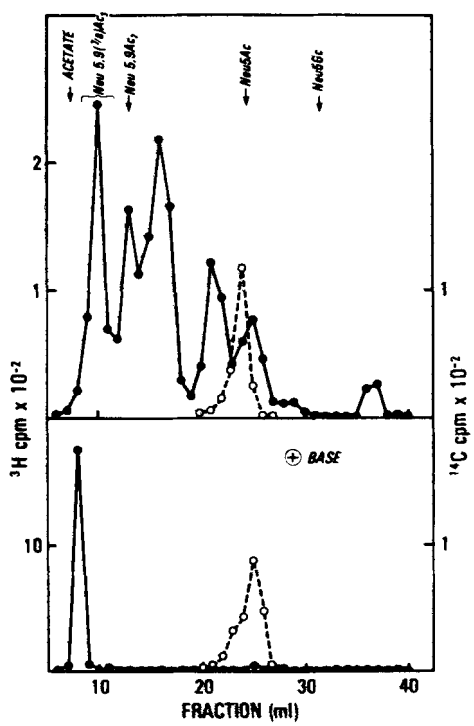

FIG. 6. **HPLC for the separation of various sialic acids.** Work was carried out exactly as described in the legend to Fig. 1. The *upper panel* shows the separation of sialic acids purified from the macromolecular fraction of Golgi vesicles labeled with [*acetyl*-^{3}H] acetyl-CoA, as described in the text. The *lower panel* shows an identical aliquot subjected to de-*O*-acetylation with 0.1 N NaOH on ice for 45 min. In each case, an internal standard of [^{14}C]Neu5Ac was added. The position of elution of free acetate and of other standard sialic acids is as indicated (compare with Fig. 1).

We therefore tried alternative approaches to their further identification. First, an aliquot of the purified labeled material was incubated for 14 h at pH 7.5 at 37 °C. Under these conditions, migration of *O*-acetyl esters along the exocyclic side chain of sialic acids, from the 7- and 8-positions to the 9-position, is known to occur (2, 35), while no significant de-*O*-acetylation occurs.[2] When the incubated label was then reapplied to the HPLC column, there was indeed very little evidence of de-*O*-acetylation (formation of free acetate). However, the profile and relative heights of the various peaks had changed significantly, strongly suggesting that migration of *O*-acetyl esters had occurred (not shown). This suggests that some of the peaks are isomers of each other with *O*-acetyl groups at different locations on the 7/8/9-side chain.

We also carried out an identical purification from Golgi vesicles labeled with CMP-[^{14}C]Neu5Ac in the presence of [^{3}H]acetyl-CoA, or CMP-[9-^{3}H]sialic acid in the presence of [^{14}C]acetyl-CoA. In such preparations, a portion of the sialic acid label (3–6%) did migrate in the positions of several of the peaks seen with the [^{3}H]acetyl-CoA preparation (data not shown). In the case of the sialic acids labeled with the CMP-[9-^{3}H]sialic acid, all of the label should be in the 9-position of the molecules. Treatment of [9-^{3}H]sialic acid with mild periodate at neutral pH (46–48) resulted in complete conversion of the ^{3}H label into [^{3}H]formaldehyde, which can be removed by evaporation.[2] However, if the sialic acids are substituted at the 9- or 8-positions with *O*-acetyl esters, the periodate reaction would be blocked (11, 49). When such periodate treatment was carried out on the purified sialic acids from the CMP-[9-^{3}H]sialic acid labeling, most of the label indeed became volatile. When the remaining nonvolatile radioactivity was reapplied to the HPLC column, it was found that the major non-*O*-acetylated [9-^{3}H]Neu5Ac peak was completely destroyed, while many of the peaks corresponding to the *O*-acetylated derivatives had survived the treatment (not shown). This further suggests that the labeled compounds are indeed *O*-acetylated sialic acids. However, these double-labeled compounds represented only 3–6% of the total labeled sialic acids, making it difficult to use them for further definitive identification. Another approach we tried was to release and purify the endogenous sialic acids of unlabeled Golgi vesicles. However, the amount of these endogenous sialic acids was very small (4.6 nmol/mg of protein). Thus, the amount of unlabeled sialic acids we obtained was also inadequate to characterize them completely by conventional methods.

Thus, although definitive identification was not possible, the various radioactive peaks seen on the HPLC analysis are very likely to be *O*-acetylated sialic acids. First, they were released by neuraminidase from the macromolecular fraction of the labeled Golgi vesicles. Second, they copurified with sialic acids through several different steps. Third, they eluted from the AX-5 HPLC column under conditions that are very characteristic for sialic acids. Last, all of the label could be converted to free acetate by base treatment, and to acetylhydroxamate by alkaline hydroxylamine treatment.

Evidence for a Golgi O-Acetyl-esterase and an Acetyl-CoA Hydrolase—During the release and purification of the [^{3}H]*O*-acetylated sialic acids, we noted that the final recovery was only 40–50% of the label that was apparently released by

neuraminidase. Under identical conditions, the recovery of ^{14}C-labeled sialic acids was 80–90%. This discrepancy was found to be due to the presence of free acetate after the neuraminidase incubation. The [^{3}H]acetate was then being lost as [^{3}H]acetic acid during the drying-down step after Dowex-50 chromatography. By incubating vesicles prelabeled with [^{3}H]acetyl-CoA or unlabeled vesicles with the [^{3}H]*O*-acetylsialic acids, we have found evidence for an esterase activity intrinsic to the Golgi membranes that is capable of cleaving *O*-acetyl groups from sialic acids.[2] The activity of this enzyme during the neuraminidase incubation probably explains the losses of label seen. As predicted by the studies described above, we have also found evidence for an acetyl-CoA hydrolase in the Golgi vesicles that can explain the extensive breakdown of the labeled acetyl-CoA during the incubations. Further studies of both activities are under way.

Characterization of Neuraminidase-resistant Radioactivity—The 15–25% of radioactivity in membranes that remained resistant to neuraminidase release was characterized further as follows. Only 10% of this label was released by neutral hydroxylamine treatment. With alkaline hydroxylamine treatment (which should release all *O*-acyl esters), only 72% of this label was released, even with repeated treatments. The product of these treatments co-migrated with acetylhydroxamate on TLC. This leaves 28% of this neuraminidase-resistant fraction resistant to alkaline hydroxylamine (or 4–7% of the original total radioactivity). We are uncertain of the nature of these various components of neuraminidase-resistant radioactivity. The neutral hydroxylamine-sensitive component could represent thiol esters. The akaline-hydroxylamine sensitive component could represent *O*-acetyl esters on neuraminidase-resistant sialic acids or on other molecules. The hydroxylamine-resistant fraction could be in other linkages, such as *N*-acetyl groups of proteins or sugars. These findings raise the possibility that the acetyl-CoA transport into the Golgi apparatus serves functions other than the *O*-acetylation of sialic acids.

DISCUSSION

In this study we have demonstrated evidence for a rat liver Golgi acetyl-CoA translocator that provides the donor for acetylation reactions within the Golgi apparatus. Surprisingly, the transporter also concentrates coenzyme ASH in the vesicles for some other unidentified donation reaction. Since CoA itself can be transported into the Golgi vesicles, it is impossible to conclusively demonstrate that the entire acetyl-CoA molecule is indeed being transported. However, it appears most likely that this is the case. The results suggest that upon entry into the Golgi lumen, the acetyl-CoA molecule is either utilized for transfer of acetyl groups, or is immediately degraded to free acetate.

The characteristics of the activity described here make it quite distinct from the acetyl-CoA utilization by rat liver lysosomes described by Rome *et al.* (24). In that case, there is no evidence for translocation of the entire molecule. The acetyl group is donated primarily to the free amino group of terminal α-linked glucosamine residues that appear during the degradation of heparan sulfate proteoglycan. Furthermore, the lysosomal process is very poorly inhibited by coenzyme ASH and is much more stimulated by ATP (24).

By several criteria this transporter also appears to be distinct from all others described to date in the Golgi apparatus (16–23, 50), including that responsible for the translocation of the structurally related molecule PAPS (20, 21, 50). In these previous studies, Pronase digestion was used to show that the transport processes in question were mediated by proteins which, at least in part, faced the exterior of the Golgi vesicles (17, 20). We were unable to carry out similar experiments because of the extreme lability of the acetyl-CoA utilization process (see "Results"). These previous studies also looked at the subcellular distribution of the sugar nucleotide transport systems and demonstrated a Golgi localization for the activities. However, unlike the case with sugar nucleotides, acetyl-CoA is known to be utilized by other organelles such as lysosomes (24) for purposes other than the *O*-acetylation of sialic acids. We therefore did not study the relative enrichment of this activity in the Golgi vesicles.

The major products found in the macromolecular fraction were *O*-acetylated sialic acids. Our data do not allow us to clearly distinguish whether the translocator function is due to a completely distinct protein from the sialic acid *O*-acetyltransferase(s). However, the fact that other (unidentified) acetylated products could be demonstrated makes this likely to be the case. It should be noted that this transport process could have a very important role in concentrating the acetyl-CoA donor to a level sufficient to drive the *O*-acetylation reaction. A previous study of a sialic acid-specific *O*-acetyltransferase in bovine submaxillary glands (15) found an apparent K_m of 100 μM. For example, cytosolic levels of acetyl-CoA that have been measured in brain tissue range from 2 to 6 μM (51). Since *O*-acetylation of sialic acids clearly occurs in brain tissue (2), the concentrating function of the acetyl-CoA transporter could be critical in allowing the reaction to proceed within the lumen of the Golgi apparatus. The marked inhibition by palmitoyl-CoA suggests that it may almost be a substrate for the translocation process. This could explain how this molecule becomes available for the addition of palmitate residues to proteins, which is known to take place in the Golgi apparatus (52).

Previous studies with bovine submaxillary glands have been interpreted to indicate that the *O*-acetylation reaction can take place either after the transfer of sialic acids to glycoconjugates or on free sialic acids in the cytosol (13–15). Our study does not provide evidence to rule out the latter possibility, but demonstrates the mechanism by which the donor molecule can be translocated into and concentrated in the Golgi apparatus for use by *O*-acetyltransferases. Since CMP-sialic acid is promptly used or hydrolyzed upon entry into the Golgi apparatus (17), it is very unlikely that it could serve as an intermediate substrate for the *O*-acetyltransferases in the Golgi lumen. It is most likely that the acetyl group is directly donated to specific sialic acids of acceptor glycoconjugates in transit through the Golgi apparatus. Recent studies have shown that the Golgi apparatus is highly organized, with the various activities involving glycoprotein biosynthesis being appropriately distributed from the cis to the trans face (52–54). If the acetyl-CoA transport process is exclusively intended for the *O*-acetylation of sialic acids, one would predict that it would be localized in the trans-most stacks, where the sialylation reactions take place.

We have provided evidence that the entire acetyl-CoA molecule is involved in the translocation process. However, the intravesicular low molecular weight fraction was found to consist almost exclusively of free acetate. We have two possible explanations for this finding. First, we have found preliminary evidence for an *O*-acetyl-esterase within the Golgi lumen that appears to be distinct from a recently described cytosolic sialic acid *O*-acetylesterase (55). Second, our data suggest the existence of an acetyl-CoA hydrolase in the Golgi vesicles. We are not sure of the relationship of this activity to previously described acetyl-CoA hydrolases (56–58). Either or both of these activities could be responsible for the gener-

ation of the free acetate found within the vesicles. We have not followed the fate of the CoA released upon utilization and/or hydrolysis of the acetyl-CoA within the Golgi lumen. Since CoA is known to be an inhibitor of the *O*-acetyltransferase(s), it would be important to eliminate it from this location in an efficient manner. It is possible that an antiport system akin to that recently described for the sugar nucleotides (23) may be involved in acetyl-CoA uptake and removal of CoA. We are currently exploring this possibility.

Thus, it appears that the *O*-acetylation of sialic acids in the Golgi apparatus could be regulated by many different processes, including an acetyl-CoA transporter, one or more sialic acid-specific *O*-acetyltransferases, an acetyl-CoA hydrolase, and an *O*-acetylesterase. In addition, some recent data suggest that there are substrate specificity factors involving the acceptor glycoconjugate itself. For example, of several gangliosides in human melanoma cells, only two were selectively *O*-acetylated (59, 60), and in a mouse brain ganglioside, only 1 of 3 sialic acid residues was found to be *O*-acetylated (61). The existence of such exquisitely tissue- and acceptor-specific *O*-acetylation reactions suggests that they must have some important biological roles.

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