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High Level *O*-Acetylation of Sialic Acids on *N*-Linked Oligosaccharides of Rat Liver Membranes

DIFFERENTIAL SUBCELLULAR DISTRIBUTION OF 7- AND 9-*O*-ACETYL GROUPS AND OF ENZYMES INVOLVED IN THEIR REGULATION*

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_O_-Acetylation of sialic acids has previously been considered an uncommon modification found on certain salivary mucins and neural gangliosides. We show here that glycosidically bound sialic acids from total membranes of rat liver have surprisingly high levels (~20%) of _O_-acetylation at the 7- or 9-position. This _O_-acetylation is further enriched in _N_-linked oligosaccharides but is barely detectable in ganglioside fractions from the same tissue. The position of _O_-acetylation on the sialic acid side chain varies between different subcellular fractions. In particular, 7-_O_-acetylation was enriched in lysosomal membranes and 9-_O_-acetylation in plasma membranes, whereas Golgi membranes contained both types. This distribution fits with the ability of the rat liver sialate:_O_-acetyltransferase(s) to synthesize both 7- and 9-_O_-acetyl esters (Diaz, S., Higa, H. H., Hayes, B. K., and Varki, A. (1989) _J. Biol. Chem._ 264, 19416–19426) and the fact that 7-_O_-acetyl esters can migrate to the 9-position at physiological temperature but only under neutral or mildly alkaline conditions. Subcellular fractionation shows that sialate:_O_-acetyltransferase activity directed toward endogenous acceptors is enriched in Golgi fractions, whereas an intralumenal sialic acid-specific _O_-acetylesterase activity is not. The O-acetyltransferase is labile and difficult to solubilize in the intact state and cannot be assayed with exogenous acceptors. However, a prelabeled [^{3}H]acetyl intermediate can be solubilized from Golgi membranes with Triton X-100 and is stable for a prolonged time in the cold. In contrast to the transferase, the lumenal esterase is easily released in a stable and water-soluble form from membrane fractions by saponin permeabilization or repeated freeze-thaw. In keeping with this finding, differential subcellular fractionation and continuous sucrose gradients indicate that this enzyme is enriched in lysosomal fractions (see also the accompanying paper (Butor, C., Higa, H. H., and Varki, A. (1993) _J. Biol. Chem._ 268, 10207–10213). Based upon findings reported in this and previous studies, a model is proposed for the biosynthesis, maturation, and turnover of 7- and 9-_O_-acetyl esters on the sialic acids of _N_-linked oligosaccharides that are attached to membrane-bound proteins in the rat liver.

Sialic acids are nine-carbon acidic sugars frequently found as the terminal units of the oligosaccharide chains on a variety of glycoconjugates (1–3). Many types of modifications of the parent sialic acid *N*-acetylneuraminic acid (Neu5Ac)[1] have been reported in nature (1–4). One of the better studied modifications is the addition of *O*-acetyl esters to hydroxyl groups at the 7–8–9-positions on the side chain of these molecules. These esters show tissue-specific and developmentally regulated expression in certain systems, and they are capable of modulating a variety of biological phenomena, such as complement activation, virus binding, bacterial sialidase action, lectin recognition, tumor antigenicity, cell adhesion, and tissue morphogenesis (2, 3). The biochemical mechanisms involved in regulating the expression of these *O*-acetyl esters are therefore of considerable interest.

The rat liver Golgi apparatus is a very well established system for the study of oligosaccharide biosynthesis. In previous studies, we reported a sialate:*O*-acetyltransferase activity in Golgi-enriched preparations from rat liver (5, 6), which was detected using [*acetyl*-^{3}H]acetyl coenzyme A (AcCoA) as a donor. The transfer reaction required intactness of the vesicles and was directed against endogenous acceptors, which consisted mainly of *N*-linked glycoproteins (5). The pattern of endogenous glycoprotein acceptors on SDS-polyacrylamide gel electrophoresis analysis was similar to that seen when labeling was carried out with CMP-[9-^{3}H]Neu5Ac. Notably, the V_{max} for transfer of label from [^{3}H]AcCoA to these endogenous acceptors (5) was about one-third of that reported by others for CMP-[9-^{3}H]Neu5Ac in the same system (7, 8). This suggested to us that *O*-acetylation might be a major modification of sialic acids in the rat liver. The rat liver sialate:*O*-acetyltransferase transfers acetyl groups directly to both the 7- and the 9-positions of the endogenous sialic acids (5). However, studies of side chain *O*-acetylation are complicated by the fact that esters at the 7-position can undergo spontaneous migration to the 9-position under physiological conditions (9, 10). Also, a family of mammalian sialate:*O*-acetylesterases have been reported that can cleave *O*-acetyl groups

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[1] The abbreviations used are: Neu5Ac, *N*-acetylneuraminic acid; Neu5,9Ac$_2$, 9-*O*-acetyl-*N*-acetylneuraminic acid; Neu5,7Ac$_2$, 7-*O*-acetyl-*N*-acetylneuraminic acid; (Neu5,7(8)9Ac$_3$, 7(8)-9-di-*O*-acetyl-*N*-acetylneuraminic acid, Neu4,5Ac$_2$, 4-*O*-acetyl-*N*-acetylneuraminic acid; DFP, diisopropylfluorophosphate; DMB, 1,2-diamino-4,5-methylenedioxybenzene; AcCoA, acetyl coenzyme A; NLOs, *N*-linked oligosaccharides; HPLC, high performance liquid chromatography.

only from the 9-position of sialic acids (11–17). In this study, we have explored the expression and distribution of *O*-acetyl esters on the side chains of sialic acids in the rat liver and the enzymes involved in their biosynthesis and turnover.

EXPERIMENTAL PROCEDURES

Materials—[*acetyl*-^{3}H]Acetyl-CoA was synthesized as described previously (17) and was determined to have a specific activity of 27 Ci/mmol. CMP-[9-^{3}H]Neu5Ac was synthesized from [6-^{3}H]ManNAc and purified as described previously (18, 19) and was determined to have a specific activity of 20 Ci/mmol. The following materials were obtained from the sources indicated: Resins AG50W-X2 (H^+ form) and AG 3X4a (formate form), Bio-Rad; *Arthrobacter ureafaciens* sialidase, Calbiochem; 1,2-diamino-4,5-methylenedioxybenzene (DMB), Sephadex G-25, G-15, and QAE-Sephadex, Sigma; and Sephacryl S-200, Pharmacia LKB Biotechnology Inc. Diisopropylfluorophosphate (DFP) from Aldrich was prepared as a 100 mM stock solution in isopropyl alcohol and stored at −20 °C. All chromatography solvents were of HPLC grade, and all other chemicals were of reagent grade. A standard mixture of *O*-acetylated sialic acids was obtained from bovine submaxillary mucin as described previously (20). Peptide:*N*-glycosidase F was purified from the culture supernatant of *Flavobacterium meningosepticum* and shown to be free of proteases and Endo-F(s) as described previously (21, 22).

Assays—Protein was measured by the method of Lowry (23). GlcNAc:β-galactosyltransferase (24), tartarate-resistant acid phosphatase (25), 5′-nucleotidase (26) (with 3′-AMP as a blank), and β-hexosaminidase (27) were assayed in subcellular fractions as described previously. Sialate:9-*O*-acetylesterase activity was assayed as described previously using [*acetyl*-^{3}H]Neu,5,9Ac$_2$ as a substrate (14). Sialyltransferase(s) directed toward *N*-linked oligosaccharides were assayed using asialo-α-1-acid glycoprotein as acceptor and CMP-[9-^{3}H]Neu5Ac as a donor. Transferred label was determined by precipitation of the protein with 5% trichloroacetic acid, 1% phosphotunastic acid.

Release and Purification of Sialic Acids—Sialic acids were released from membranes or other samples by incubation with 5–10 milliunits of *A. ureafaciens* sialidase in 100 μl of 100 mM sodium acetate, pH 5.5, containing 0.5% Triton X-100 for 14–16 h at 37 °C under a toluene atmosphere. Released sialic acids were purified by ion exchange chromatography on Bio-Rad AG50 1x8 (hydrogen form) followed by Bio-Rad AG3x4A (formate form) as described previously (5). These purification conditions have been shown previously to avoid destruction of *O*-acetyl esters and limit migration of 7-*O*-acetyl esters to less than 10% (5).

Preparation of Sialic Acids from Different Glycoconjugate Fractions of Rat Liver—Male Sprague-Dawley rats (3–5 months in age) were anesthetized with ether and decapitated. The livers were flushed out with phosphate-buffered saline, pH 6.5, removed, finely minced, and homogenized with a Polytron in 0.5 M sucrose, 50 mM sodium maleate, pH 6.5 (24 ml for 8 g of liver). The suspension was spun at 650 × *g* for 10 min and the supernatant collected. The pellet was resuspended in 1 ml of homogenization buffer/g of liver and spun at 650 × *g* for 5 min. Supernatants were pooled (postnuclear supernate), DFP added to 1 mM, and the sample incubated on ice for 30 min. The postnuclear supernate was diluted 1/20 with ice-cold water, adjusted to 1 mM EDTA, and the hypotonically lysed membranes spun for 30 min at 100,000 × *g*. The pellet was resuspended in 1 M NaCl and spun as above. The salt-washed pellet was resuspended in 50 mM Tris/HCl, pH 6.5, and re-pelleted (washed membranes). A portion of the washed membranes (for total sialic acids) was resuspended in sialidase buffer and the sialic acids released with sialidase (see above). Another aliquot (for sialic acids on *N*-linked oligosaccharides) was treated with peptide:*N*-glycosidase F as described below. A third aliquot (for lipid-linked sialic acids) was resuspended in small quantity of 50 mM Tris/HCl, pH 6.5, and lyophilized. The dried residue was extracted sequentially with 1 ml each of chloroform/methanol, 2:1, 1:1, and 1:2 and then with 1 ml of chloroform/methanol/water, 10:10:1. The pooled extracts were dried, and the dried residue was re-extracted with 1 ml of chloroform/methanol (1:1). Under these conditions of lipid extraction, *O*-acetyl groups are known to be stable (17, 28). The final extract was dried down, sonicated into 100 μl of 0.5% sodium taurocholate in 100 mM sodium acetate, pH 6.0, and treated with *A. ureafaciens* sialidase alongside the other samples.

Release and Purification of N-Linked Oligosaccharides—*N*-Linked oligosaccharides from total rat liver membrane glycoproteins were released and purified as described previously with small changes (see Ref. 29 for full details). Briefly, the washed membranes were solubilized by heating in 50 mM Tris/HCl, pH 6.5, 0.1 M 2-mercaptoethanol, and 1% SDS at 100 °C for 10 min and the lysate clarified by centrifugation at 100,000 × *g* for 30 min. The supernate was loaded onto a 0.7 × 50-cm Sephacryl S-200 column run in 0.2% SDS, and the void volume fractions were collected and acetone-precipitated. The precipitate was resuspended in 200 μl of 50 mM Tris/HCl, pH 6.5, 0.5% SDS, 30 mM 2-mercaptoethanol and boiled for 5 min. The sample was cooled, Nonidet P-40 (2.5% final) was added and the lysate incubated with 6.7 Genzyme units of peptide:*N*-glycosidase F at 37 °C overnight. The digest was passed over the same S-200 column, and the included (released) fractions containing oligosaccharides were monitored for sialic acids by the thiobarbituric acid assay (30). The released chains were pooled, KCl-precipitated to remove SDS, passed over a Bio-Beads SM-2 column, and desalted on a P-2 column in H_2O.

Incorporation of Label from [^{3}H]AcCoA and CMP-[^{14}C]Neu5Ac into Sialidase-sensitive Molecules—When these nucleotides are incubated with membrane fractions in the absence of detergent, label is incorporated into endogenous acceptors (5, 31), which are completely resistant to exogenously added proteases.[2] Aliquots of the membrane fractions were washed, resuspended, and incubated for 15 min on ice in PKM (10 mM KP_i (potassium phosphate buffer), 150 mM KCl, 1 mM $MgCl_2$, pH 7.0) with 1 mM DFP (to inactivate endogenous esterases that can destroy *O*-acetyl groups). The membrane vesicles were then brought to room temperature and incubated with 10 μM [^{3}H]acetyl-CoA and/or 5 μM CMP-[^{14}C]Neu5Ac in PKM in a total volume of 200 μl for 5 min. Reactions were quenched with 3 ml of ice-cold PKM and the vesicles pelleted at 100,000 × *g* for 30 min. The supernate was aspirated and the pellet carefully surface washed three times by filling the tube with ice-cold PKM followed by aspiration. The labeled membranes were suspended in 250 μl of 100 mM sodium acetate, 0.5% Triton X-100, pH 5.5, and incubated with 20 milliunits of *A. ureafaciens* sialidase at 37 °C for 1 h. The release of radioactive sialic acids was monitored by precipitation of the reactions with 4% perchloric acid and counting of the acid-soluble fraction as described previously (5, 8). Background values to be subtracted were from identically incubated sample blanks in which the sialidase was left out.

HPLC Analysis of Released Sialic Acids—Sialidase-released sialic acids were purified, derivatized with DMB, and analyzed by reverse-phase HPLC on a TSK-ODS 120T column as described (32, 33), except that a shallow gradient was used for elution, to effect better separation of the sialic acid derivatives from reagent peaks (CH_3CN:50% MeOH:H_2O, starting at 7:14:79, and changing to 11:14:75 over 40 min). Derivatization with DMB was done before and after induced migration or de-*O*-acetylation (5) and the changes in peak area monitored to confirm the identification of the different sialic acids. In a few cases, reagent peaks partly overlapped with *O*-acetylated sialic acid peaks. In these instances, the change in the combined peak area after de-*O*-acetylation was used to calculate the amount of the sialic acid. When appropriate, material purified from sham incubations (without sialidase) was also studied to identify spurious peaks.

Subcellular Fractionation of Golgi, Plasma Membranes, and Lysosomes—In studies to localize the intralumenal (lysosomal) sialic acid-specific 9-*O*-acetylesterase activity, a single batch of rat liver was used to separately prepare Golgi (34, 35), plasma membranes (36), and lysosomes (25) by the originally described methods, with the following minor modifications; for the plasma membrane prep, the homogenate was centrifuged at 1000 rpm in a clinical centrifuge, and the supernatant was used as the total homogenate; for the lysosome prep, the initial homogenization was with a Polytron rather than a Dounce, and the initial nuclear pellet was resuspended once and the two supernatants combined. In these studies, typical enrichment of marker enzymes were ~40-fold for sialyltransferase(s) (Golgi), ~10-fold for 5′-nucleotidase (plasma membranes), and about ~10-fold for acid phosphatase (in crude lysosomes).

Subcellular Fractionation by Continuous Sucrose Gradients—Freshly collected rat liver (7 g) was minced and homogenized with a Polytron in either 20 ml of 0.25 M sucrose, 50 mM sodium maleate, pH 6.5, or 20 ml of 0.25 M sucrose, 20 mM Tris/HCl, pH 7.4. The preparations were then spun at 600 × *g* for 10 min and ~15 ml of the supernatants layered on a 20-ml continuous sucrose gradient (0.5–1.5 M) which was poured over a 1-ml 2 M sucrose cushion (all in the same

[2] B. K. Hayes and A. Varki, submitted for publication.

buffer as the initial homogenate). The gradients were centrifuged for 18 h at 100,000 × *g* at 4 °C. Fractions (~0.8–1.0 ml) were collected from the bottom of the tube via a glass capillary.

Simultaneous Preparation of Golgi Membranes, Plasma Membranes, and Lysosomal Membranes for Sialic Acid Analysis—Previously described methods for the selective isolation of subcellular fractions enriched in Golgi (34, 35), plasma membranes (36), and lysosomes (25) were adapted into a combined protocol, starting with a single rat liver homogenate. All buffers were adjusted to pH 6.5 throughout all steps to protect *O*-acetylated sialic acids from migration or de-*O*-acetylation. Initial homogenization with a Polytron was done in 0.5 M sucrose, 50 mM sodium maleate with 1 ml/g liver. After spinning at 600 × *g* for 10 min, the pellet was re-homogenized with the same buffer and spun at 650 × *g* for 5 min. Both supernates were pooled (postnuclear supernate) and split into three aliquots for preparation of Golgi membranes, plasma membranes, and lysosomal membranes.

One aliquot (for the Golgi fraction) was adjusted to 5 mM $MgCl_2$ and 20-ml aliquots layered on 8-ml 1.3 M sucrose cushions. After spinning at 63,000 × *g* for 120 min, the crude smooth membranes were collected at the interface, adjusted to 1.1 M sucrose (monitored with a densitometer), and layered between 8 ml of 1.25 M sucrose and 7 ml of 1.0 M sucrose in 50 mM sodium maleate. The tube was filled with 5 ml of 0.5 M sucrose and spun at 25,000 rpm in a Beckman SW27 rotor for 90 min. The Golgi vesicles were collected at the 0.5–1.0 M sucrose interface and adjusted to 0.25 M sucrose and 0.4 M NaCl. Half of the sample was treated with 1 mM DFP. Both sets of samples were spun at 165,000 × *g* for 30 min. The pellet from the untreated sample was resuspended in 0.25 M sucrose, 20 mM KP_i, pH 7.0, and frozen at −20 °C (for enzyme assays). The pellet from the DFP-treated sample was resuspended in 0.025 M sucrose (2 ml/g of liver) and allowed to undergo hypotonic lysis on ice for 30 min. The sample was then spun at 165,000 × *g* for 30 min. The pelleted membranes were resuspended in 1 M NaCl and spun at 165,000 × *g* for 30 min. Excess salt was removed by resuspending in sialidase buffer and spinning again at 165,000 × *g* for 30 min. The final pellet was resuspended in sialidase buffer for treatment.

Another aliquot of the postnuclear supernate (for the plasma membrane fraction) was diluted with 1 volume of 40 mM Tris/HCl, pH 6.5, and 1 volume of 0.25 M sucrose 20 mM Tris/HCl, pH 6.5. It was then overlayed on 15 ml of 1.45 M sucrose, 20 mM Tris/HCl, pH 6.5 (original method makes an underlay) and spun at 35,000 × *g* for 30 min. The crude plasma membrane fraction at the interface was recovered with a Pasteur pipette and diluted 4-fold with buffered 0.25 M sucrose. This was spun at 7000 × *g* for 15 min. The pelleted washed membranes were resuspended in buffered 0.25 M sucrose (1 ml/10 g of original liver) manually. Thirteen ml of sucrose-Percoll (prepared by thoroughly mixing 5.4 ml of Percoll with 22 ml of 0.31 M sucrose in 25 mM Tris/HCl, pH 6.5) was added to 0.8 ml of these washed membranes and the mixture spun at 33,600 × *g* for 30 min. The upper band was collected with a Pasteur pipette and diluted 30-fold with cold TBS. The membranes were spun at 1460 × *g* for 5 min and the pellets resuspended in 50 mM Tris/HCl, pH 7.4. DFP (1 mM final) was added to half of the preparation and the membranes pelleted in a microfuge during 1 min. The untreated membranes (for enzyme assays) were pelleted, resuspended in 50 mM Tris/HCl, pH 7.4, and stored frozen at −20 °C. The DFP-treated pellet was resuspended in 0.025 M sucrose (2 ml/g of liver) and allowed to undergo hypotonic lysis on ice for 30 min. The sample was then spun at 165,000 × *g* for 30 min. The pelleted membranes were resuspended in 1 M NaCl and spun at 165,000 × *g* for 30 min. Excess salt was removed by resuspending in sialidase buffer and spinning again at 165,000 × *g* for 30 min. The final pellet was resuspended in sialidase buffer for treatment.

Another aliquot of the postnuclear supernate (for lysosome fraction) was diluted with 1 volume of 0.4 M KCl, pH 6.5, and spun at 11,000 × *g* for 20 min. The pellet was resuspended in half the volume of the diluted homogenate in buffered 0.25 M sucrose solution and spun at 11,000 × *g* for 30 min. The pellet (mitochondrial/lysosome fraction) was resuspended in 0.025 M sucrose (2 ml/g of liver) and allowed to swell for 30 min on ice. The membranes were spun at 11,000 × *g* for 20 min and the supernatant saved for the esterase assay. The pellet was resuspended in 0.025 M sucrose, 0.02 M KCl (1 ml/g of liver) and spun for 30 min at 11,000 g. The supernatants were pooled (crude lysosomal membrane fraction), adjusted to 10 mM $CaCl_2$, and allowed to swell on ice for 30 min. The preparation was spun 10 min at 1500 × *g*. The supernatant was spun 10 min at 5000 × *g*. The supernatant was spun 30 min at 10,000 × *g*. The last pellet lysosomal membranes was resuspended in 1 M NaCl. 1 mM DFP was added to half of each fraction. Both aliquots were spun at 105,000 × *g* for 60 min. The untreated pellets were resuspended in phosphate-buffered saline for enzyme assays and stored frozen at −20 °C. The DFP-treated pellets were resuspended in sialidase buffer, spun at 165,000 × *g* for 30 min, and resuspended in sialidase buffer for treatment.

Typical enrichment of marker enzymes in the final membrane preparations were ~60-fold for GlcNAc:β-galactosyltransferase (Golgi), ~10-fold for 5′-nucleotidase (plasma membranes), and about ~60-fold for acid phosphatase (lysosomal membranes). The sialic acids were released from each fraction, further purified, and analyzed by HPLC as described above.

RESULTS

Rat Liver Membranes Have High Levels of O-Acetylation of Sialic Acids—The extent of *O*-acetylation of glycosidically bound sialic acids in freshly isolated rat liver membranes was examined. Total cellular membranes were isolated and subjected to hypotonic lysis and washing with high salt, to remove soluble and peripherally associated proteins. Sialic acids were released from these washed membranes by the sialidase from *A. ureafaciens* (*A. ureafaciens* sialidase), which can cleave unsubstituted and side chain *O*-acetylated sialic acids from all known linkages (9). The released sialic acids were purified by methods designed to minimize de-*O*-acetylation or *O*-acetyl migration (5, 33), derivatized with DMB, and studied by reverse-phase HPLC as described under "Experimental Procedures." As a comparative standard (not shown), sialic acids were also released from bovine submaxillary mucin, which contains a variety of substituted sialic acids (33, 37). As shown in Fig. 1, the rat liver membrane sialic acids included Neu5Ac, small amounts of *N*-glycolylneuraminic acid (Neu5Gc), and several peaks whose elution position was consistent with the presence of one or more *O*-acetyl groups. Treatment with dilute ammonia under controlled conditions that cause migration of *O*-acetyl esters from the 7- to the 9-position (5) resulted in loss of the peak corresponding to 7-*O*-acetyl-*N*-acetylneuraminic acid ($Neu5,7Ac_2$), and an increase of the peak corresponding to 7(8)-9-di-*O*-acetyl-*N*-acetylneuraminic acid ($Neu5,7(8)9Ac_3$). Some overall de-*O*-acetylation is unavoidable during this chemically induced migration. Treatment with stronger base conditions designed to give complete de-*O*-acetylation (33) caused a disappearance of the peaks corresponding to 7- and 9-*O*-acetyl-*N*-acetylneuraminic acid ($Neu5,9Ac_2$) and $Neu5,7(8)9Ac_3$. (The latter overlaps with a reagent peak and is therefore quantitated by subtraction). There was a corresponding increase in the peak area for Neu5Ac. Taken together, the data indicate the presence of substantial amounts of *O*-acetylation (in the range of 15–25% for various experiments). Interestingly, there was no increase in the peak area for Neu5Gc after de-*O*-acetylation, indicating that this sialic acid is not *O*-acetylated to the same extent as Neu5Ac. Two small additional base-labile peaks were noted (see Fig. 1, *peaks 4* and *5*), whose identity is not clear. These peaks were not found in material purified from control incubations without added sialidase (not shown), indicating that they do represent glycosidically bound sialic acids released from the rat liver membranes. Their identity is not clear at present, and they will be referred to hereafter as peak 4 and peak 5. It should be noted that 4-*O*-acetylated sialic acids would have been resistant to all available sialidases (12, 38, 39) and hence would not be detected in this study.

O-Acetylation Is Enriched in N-Linked Oligosaccharides—In previous studies, we reported that when [*acetyl*-^{3}H]AcCoA was incubated with isolated intact rat liver Golgi-enriched vesicles (5, 7, 8), label was transferred to endogenous acceptors at a V_{max} that was about one third of that seen for label from CMP-[^{3}H]Neu5Ac. In both cases, most of the radioactivity

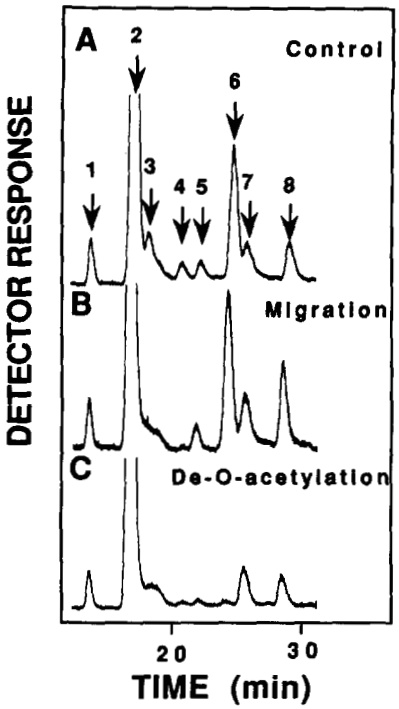

FIG. 1. **Analysis of rat liver membrane sialic acids by HPLC after DMB derivatization.** Total membranes from a postnuclear supernate of rat liver were hypotonically lysed, salt washed, and the sialic acids released, purified, derivatized, and analyzed as described under "Experimental Procedures." Purified sialic acids were derivatized before or after induced migration or complete de-*O*-acetylation. The *arrows* mark the position of elution of Neu5Gc (*arrow 1*), Neu5Ac (*arrow 2*), $Neu5,7Ac_2$ (*arrow 3*), unknown sialic acids (*arrows 4* and *5*), $Neu5,9Ac_2$ (*arrow 6*), reagent peak (*arrow 7*), and $(Neu5,7(8)9Ac_3$, partly overlapping with another reagent peak (*arrow 8*). Authentic sialic acid peaks were also identified by studying sialic acids released from bovine submaxillary mucin and by monitoring the elution profile of material purified from sham incubations without sialidase (profiles not shown).

was found in a family of endogenous glycoproteins and the labeled bands on SDS-polyacrylamide gel electrophoresis were markedly diminished by treatment with either *A. ureafaciens* sialidase or peptide:*N*-glycosidase F (5). This *in vitro* study suggested that the *O*-acetylation reaction might be primarily directed at *N*-linked oligosaccharides (NLOs) and that the extent of *O*-acetylation in this fraction might be high. To explore this matter in the native tissue, freshly prepared washed rat liver membranes were subjected to either lipid extraction (for selective extraction of gangliosides) or to lysis in SDS, followed by specific release of NLOs with peptide:*N*-glycosidase F, as described under "Experimental Procedures." Sialic acids were released from the purified NLOs, the gangliosides, or the starting total membranes, purified, derivatized, and analyzed as described under "Experimental Procedures." Previous studies have shown that under all of these conditions of handling, loss of *O*-acetyl groups is minimal (5). However, because of the need to boil samples for peptide:*N*-glycosidase F treatment, some migration of *O*-acetyl groups is likely in some of the samples. The analysis was therefore directed only toward obtaining data on overall levels of *O*-acetylation in each fraction. As shown in Table I and Fig. 2, *O*-acetylation was enriched in the NLO fraction and markedly depleted in the ganglioside fraction. Since the "ganglioside fraction" consisted of a total lipid extract without further purification, it is possible that even the small amount of *O*-acetylation seen here is due to residual contaminating proteins. Although current technology does not allow direct analysis for *O*-acetyl groups on sialic acids of *O*-linked oligosaccharides, the lipid extract and NLOs appear to account for most of the sialic acids in the membrane fraction (see Table I). Thus, it appears that most, if not all, of the *O*-acetylated sialic acids in the total membrane fraction may be on the NLOs. Also, the lack of *O*-acetylation on the Neu5Gc molecules noted in the total membranes was again seen in the NLOs (data not shown).

The Distribution of 7-O- and 9-O-Acetylation Varies between Different Subcellular Fractions—7-*O*-Acetyl esters on the side chain of sialic acids can migrate to the 9-position (if the latter hydroxyl group is unoccupied) under certain conditions of pH and temperature (9, 10). On the other hand, all sialate *O*-acetylesterases reported to date work only on *O*-acetyl esters at the 9-position. To see if the distribution of 7-*O*- and 9-*O*-acetylation on sialic acids correlated with the different pH conditions expected in different parts of the cell, we isolated from a single homogenate of rat liver, subcellular fractions that were highly enriched in Golgi vesicles, plasma membranes, or lysosomes. Throughout all steps, the pH (6.5) and temperature (0–4 °C) were maintained such that migration of *O*-acetyl esters could not occur. The homogenate was also treated at the outset with DFP a membrane-permeant inactivator of endogenous 9-*O*-acetylesterases. The different membrane fractions were exposed to hypotonic lysis, washed, and the sialic acids released and purified using methods that allow <2% loss and <10% migration of *O*-acetyl esters (5). As shown in Table II and Figs. 3 and 4, 9-*O*-acetylation was preferentially enriched in plasma membrane fractions, whereas 7-*O*-acetylation was enriched in lysosomal membranes and Golgi-enriched fractions contained both types in a ratio that varied between preparations.

O-Acetyltransferase Activity Is Enriched in Golgi Fractions, Whereas O-Acetylesterase Activity Is Not—We have shown previously that when [*acetyl*-^{3}H]AcCoA is incubated with rat liver Golgi-enriched vesicles, label is transferred to endogenous acceptor glycoproteins (5) and that an endogenous DFP-sensitive sialate:*O*-acetylesterase activity is able to remove the labeled *O*-acetyl groups from these acceptors (5). Although these studies demonstrated the presence of these activities, they did not identify their major subcellular localization. For most glycosyltransferases, the next step in exploring this issue is the setting up of a quantitative assay using exogenous acceptors and detergent (40). However, in this case we have been unable to detect the *O*-acetyltransferase activity with exogenous macromolecular acceptors after membrane solubilization. We therefore followed the *O*-acetyltransferase activity against *endogenous* acceptors (in the absence of detergent) to see if it was selectively enriched in the Golgi. As shown in Tables III and IV, this proved to be the case. Since AcCoA is involved in a large number of reactions in a variety of subcellular sites, only a small fraction (<5%) of the label transferred from [*acetyl*-^{3}H]AcCoA in postnuclear supernates was found in sialic acids. However, this fraction increased to 38% in crude smooth membranes and was enriched further to 75% in the Golgi fraction. Although very little of the label transferred to total cellular membranes from [*acetyl*-^{3}H]AcCoA was found in the Golgi (0.6%), the latter was predominantly in sialic acids. Of course, this transferase reaction against endogenous acceptors is not strictly quantitative, since the acceptor concentration may be nonsaturating and could potentially be affected by unknown endogenous factors in the different fractions. However, it is notable that the values obtained for -fold purification and yield of the *O*-acetylation of endogenous acceptors are similar to those for GlcNAc:β-galactosyltrans-

TABLE I

Recovery and extent of O-acetylation of sialic acids in N-linked oligosaccharides and gangliosides from rat liver

Total membranes, total lipid extracts, or purified *N*-linked oligosaccharides were prepared from parallel aliquots of a postnuclear supernate of rat liver as described under "Experimental Procedures." Sialic acids from each fraction were released, purified, and analyzed as described under "Experimental Procedures." The unknown minor base-labile peaks (4 and 5) were not included in the calculations presented in this table.

Sample	Total sialic acid		*O*-Acetylated sialic acid		
	nmol/ml[a]	Recovery	nmol/ml[a]	*O*-Acetylation	Recovery
		%		%	%
Total membranes	3.42	100	0.5	15	100
Lipid extract	2.5	73[b]	0.05	≤2	≤10
N-Linked oligosaccharides	1.26	37[b]	0.34[c]	27[c]	68[c]

[a] Calculated per ml of total starting postnuclear supernate.

[b] Sialidase release is likely to be more efficient on free oligosaccharides or gangliosides than on crude membrane fractions. This may account for the small over-recovery in the two fractions.

[c] The methods used for release and purification of *N*-linked oligosaccharides require boiling twice at pH 6.5, which could cause slight de-*O*-acetylation. These values may therefore be underestimates.

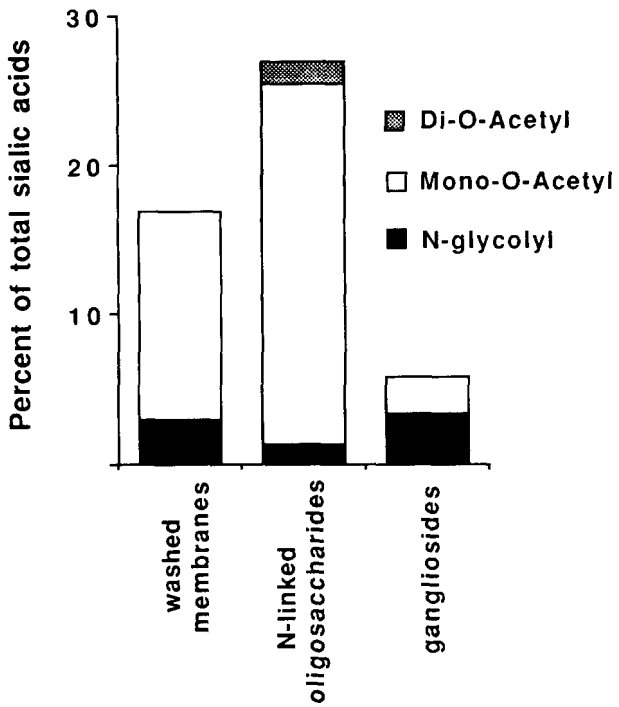

FIG. 2. **Differential distribution of *O*-acetylation of sialic acids between different types of oligosaccharides.** The percentage of sialic acids other than *N*-acetylneuraminic acid are presented in this graph. Experimental details are presented in Table I. Because some migration of *O*-acetyl esters could have occurred during the preparation of the *N*-linked oligosaccharides, the data for total *O*-acetylation is presented, without details of the specific types of *O*-acetylation. The minor unknown base-labile peaks (*peaks 4* and 5) were not included in calculating the data presented in this figure.

ferase, a classical Golgi marker (see Table III). In striking contrast, the 9-*O*-acetylesterase activity contained within membranes, although detectable in the Golgi, was not substantially enriched in this fraction.

Since sialylation must occur before *O*-acetylation (5), we compared the incorporation of label from AcCoA and CMP-Neu5Ac into endogenous acceptors. Freshly isolated total membrane vesicles, crude smooth vesicles, and Golgi-enriched fractions prepared as above were incubated with [^{3}H]AcCoA and CMP-[^{14}C]Neu5Ac for a short time. After re-isolating the vesicles, sialidase-releasable radioactivity from endogenous acceptors was quantitated, in comparison with controls incubated without sialidase. As shown in Table IV, the incorporation of radioactivity from [^{3}H]AcCoA into sialic acids showed a distribution similar to that for CMP-[^{14}C]Neu5Ac, with a substantial enrichment and similar recovery in the Golgi fraction.

The O-Acetyltransferase Reaction Co-migrates with Golgi Markers on Sucrose Density Gradients of Rat Liver Membranes—Co-enrichment during subcellular fractionation implies co-localization. An alternate and perhaps better criterion is co-sedimentation in continuous sucrose density gradients. Total rat liver postnuclear supernates were prepared in isotonic sucrose, loaded onto continuous sucrose gradients, and centrifuged to equilibrium (18 h). Fractions were then collected and monitored for the [*acetyl*-^{3}H]AcCoA *O*-acetyltransferase activity against endogenous acceptors and for other conventional markers. Gradients were prepared in different types of buffers, with and without 5 mM Mg^{2+} ions. Similar results were obtained in all cases, and those obtained in maleate buffer pH 6.5 are presented in Fig. 5. It can be seen that the *O*-acetyltransferase activity against endogenous acceptors gives a very similar profile to that for the Golgi marker, GlcNAc:β-galactosyltransferase, and a very distinct profile from that for the markers of endoplasmic reticulum, lysosomes, and plasma membranes. The caveats mentioned in the previous section regarding the endogenous *O*-acetyltransferase activity are relevant to this experiment as well. However, these results further strengthen the notion that the *O*-acetyltransferase is *functionally* localized to the Golgi apparatus and/or closely related organelles.

The O-Acetyltransferase Is Labile and Difficult to Solubilize in a Functional State—We have reported previously that solubilization of the Golgi vesicles with Triton X-100 results in loss of transfer of *acetyl*-^{3}H groups to endogenous acceptors (5, 6). This phenomenon was reproduced with several other detergents, including octyl glucoside, CHAPS, taurocholate, and deoxycholate. On the other hand, we found that the transferase could not be assayed with exogenous sialoglycoprotein acceptors such as fetuin under a variety of conditions (data not shown). We have also reported previously that when Golgi vesicles prelabeled with [*acetyl*-^{3}H]AcCoA are permeabilized with saponin, a membrane-associated [^{3}H]acetyl intermediate can be detected that can transfer label to high (10–20 mM) concentrations of Neu5Ac but not to similar concentrations of glucuronic acid (6). Using Triton X-100 to extract prelabeled vesicles, we have now found that this [^{3}H]acetyl intermediate can be solubilized from Golgi membranes and is stable for several days at 4 °C (data not shown). However, attempts at further purification of this labeled intermediate using a variety of methods led to loss of transferable label.

TABLE II

Distribution of 7- and 9-O-acetylation in the membrane sialic acids of different subcellular organelles of rat liver

From single postnuclear supernate of rat liver, parallel techniques were used to enrich the different types of subcellular fractions as described under "Experimental Procedures." Aliquots of each membrane fraction were subjected to hypotonic lysis, followed by a high salt wash. Sialic acids were immediately released and purified from the washed membranes and derivatized and analyzed by HPLC as described under "Experimental Procedures." The decrease in the areas of the individual sialic acid peaks after base treatment (de-O-acetylation, see "Experimental Procedures") was summed up to obtain the value of total percent base-sensitive. The two minor base-labile peaks (4 and 5) are unknown sialic acids (found only in sialidase-treated samples and not in blanks) and are also included in this calculation.

	% base-sensitive	% total sialic acids						
		Neu5Ac	Neu5Gc	$Neu5,7Ac_2$	$Neu5,9Ac_2$	$Neu5,7(8)9Ac_3$	Peak 4	Peak 5
Total membranes	26.86	70.45	2.69	2.99	16.05	3.69	1.96	2.17
Golgi apparatus	23.96	73.15	2.89	3.72	16.58	1.24	0.73	1.69
Plasma membrane	29.25	70.75	3.78	0.00	20.89	1.81	0.83	1.94
Lysosomes	20.81	76.31	2.87	11.26	6.53	0.83	1.05	1.14

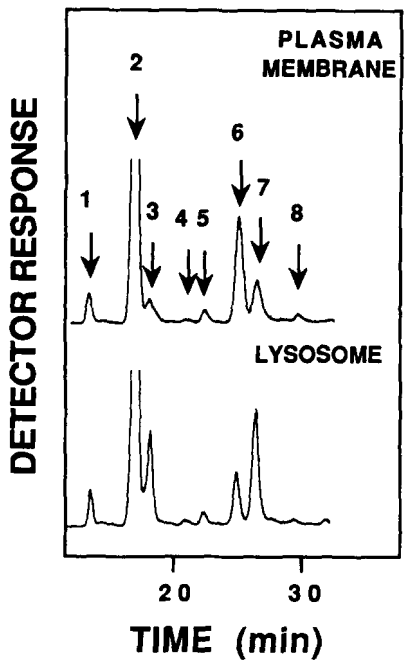

FIG. 3. **HPLC analysis of sialic acids in plasma membrane and lysosomal membrane fractions from rat liver.** Subcellular fractions were purified, membranes were hypotonically lysed and salt-washed, and the sialic acids released, purified, derivatized, and analyzed as described under "Experimental Procedures." The *arrows* mark the position of elution of Neu5Gc (*arrow 1*), Neu5Ac (*arrow 2*), $Neu5,7Ac_2$ (*arrow 3*), unknown sialic acids (*arrows 4* and *5*), $Neu5,9Ac_2$ (*arrow 6*), reagent peak (*arrow 7*), and $Neu5,7(8)9Ac_3$, partly overlapping with another reagent peak (*arrow 8*).

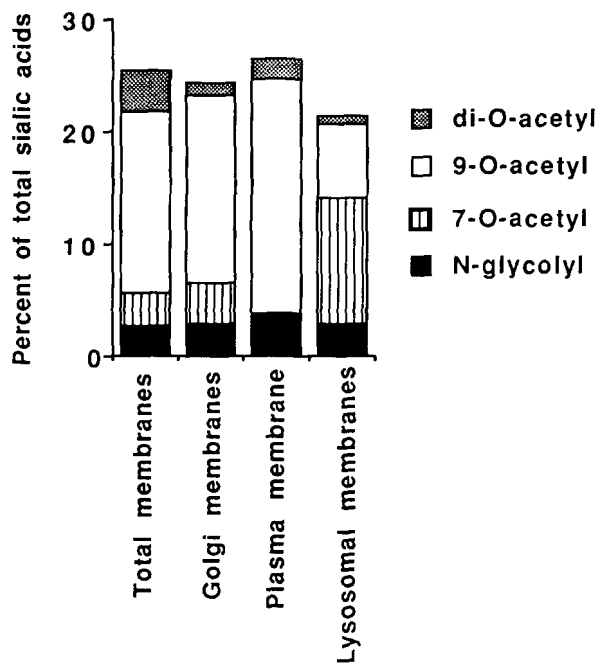

FIG. 4. **Differential distribution of 7- and 9-O-acetylation in the membrane-associated sialic acids of different subcellular organelles.** The percentage of sialic acids other than *N-acetylneuraminic acid are presented in this graph.* Experimental details are presented in Table II. The minor unknown base-labile peaks (*peaks 4* and *5*) were not included in calculating the data presented in this figure.

Thus, further purification of the transferase could not be attempted.

Permeabilization or Freeze-Thaw Causes Release of Most of the Membrane-associated Esterase—About half of the total sialate:9-O-acetylesterase activity in rat liver homogenates stays in the supernate after high-speed centrifugation, and very little of this "cytosolic" activity binds to concanavalin A-Sepharose (data not shown). This is presumed to represent the cytosolic sialate:O-acetylesterase described previously in other tissues (12, 13, 41). The other half of the esterase activity in the homogenate is pelleted down with the membrane fraction. However, we found that ~85% of this membrane-associated activity can be released by three to four cycles of freeze-thaw or after permeabilization with 0.02% saponin. The activity released is stable, freely water-soluble, and almost quantitatively bound to concanavalin A-Sepharose, indicating that it is in a glycoprotein with *N*-linked oligosaccharides. Purification of this lumenal esterase activity shows that it is identical to the concanavalin A binding sialic acid esterase that we have earlier purified from rat liver acetone powder (14, 16).

The Membrane-associated Esterase Is Intralumenal and Co-fractionates with Lysosomal Markers—In the sucrose gradient fractionations of rat liver homogenates described above, the membrane-associated 9-O-acetylesterase activity did not co-migrate with the Golgi marker. Rather, under all conditions studied, two broad peaks were seen, one which corresponds to the soluble cytosolic form at the top of the gradient and another spread through the bottom of the gradient, which presumably represents the lumenally restricted enzyme (see Fig. 6 for an example). The lower peak co-migrated best with the lysosomal marker in all such gradients. This is somewhat surprising, considering that the purified enzyme has a neutral pH optimum, with little activity below pH 5.5 (14). To further explore this matter, we carried out subcellular fractionation studies of rat liver using techniques specifically designed to enrich for lysosomes and for other organelles that could

TABLE III

Subcellular fractionation to follow enrichment of the sialate:O-acetyltransferase and sialate 9-O-acetylesterase

Enzyme activities, protein concentrations, and the incorporation of [^{3}H]AcCoA into sialidase-sensitive radioactivity were measured as described under "Experimental Procedures." The recoveries of each marker are shown using either the total homogenate or the crude smooth membrane fraction as 100% (the latter gives a value for the step recovery from the crude smooth membranes to the lysosomes).

		Postnuclear supernate	Crude smooth membranes	Golgi-enriched fraction
Recovery (%)	[*acetyl*-^{3}H]AcCoA uptake	100%	2.6%	0.6%
			100%	23%
	GlcNAc:β-galactosyltransferase	100%	95.0%	74.4%
			100%	70.0%
	Sialate:9-*O*-acetylesterase	100%	51.0%	4.0%
			100%	7.8%
Enrichment (-fold)	GlcNAc:β galactosyltransferase	1-Fold	5-Fold	67.6-Fold
	9-*O*-acetylesterase	1-Fold	2.7-Fold	3.8-Fold
[*acetyl*-^{3}H] label	% label sialidase-sensitive	<5%[a]	38%	74.5%

[a] In this experiment the background on the sialidase-minus blank with the postnuclear supernatant was too high to permit accurate subtraction.

TABLE IV

Sialidase-releasable radioactivity from endogenous acceptors in rat liver subcellular fractions labelled with [^{3}H]AcCoA and CMP-[^{14}C]Neu5Ac

Enzyme activities, protein concentrations, and the incorporation of label from [^{3}H]AcCoA and CMP-[^{14}C]Neu5Ac into sialidase-sensitive radioactivity were measured as described under "Experimental Procedures."

	Postnuclear supernatant	Crude smooth membranes	Golgi-enriched fraction
Total protein (mg)	24,256	5,030	145
cpm/min/mg transferred			
^{3}H	2,815	10,479	158,509
^{14}C	52	116	1,884
-Fold purification			
^{3}H	1-Fold	3.7-Fold	56.3-Fold
^{14}C	1-Fold		36.2-Fold
Total cpm transferred per min			
^{3}H	68,250,240	52,708,320	22,983,840
^{14}C	1,272,640	583,200	273,240
Recovery (%)			
^{3}H	100%	77.2%	33.7%
^{14}C	100%	45.8%	21.5%

contain sialylated substrates for the enzyme. As shown in Table V, the membrane-associated 9-acetylesterase activity again co-purified with a standard lysosomal marker and not with markers for endoplasmic reticulum, Golgi, or plasma membranes.

DISCUSSION

Membrane vesicles from rat liver homogenates could carry sialic acids on soluble proteins within their lumen or on glycoconjugates directly associated with the membranes. The former should include lumenal proteins such as lysosomal enzymes and secreted sialoglycoproteins in transit through the Golgi apparatus, whereas the latter should include gangliosides and membrane-bound glycoproteins. Although rat serum glycoproteins are known to carry some *O*-acetylated sialic acids (2), a detailed study of the matter has not been carried out. In this study, the extent of *O*-acetylation of the sialic acids on membrane-bound glycoproteins in rat liver was examined for the first time and found to be quite high. It is noteworthy that in a tissue as well studied as the rat liver, the presence of substantial levels of *O*-acetylation of sialic acids has hitherto been missed. In fact, a variety of technical limitations make it likely that similar levels of *O*-acetylation may have been missed in many other systems by investigators studying sialoglycoconjugates. These *O*-acetyl esters are rather labile and can alter the behavior of sialic acids during release, purification, and analysis. *O*-Acetylation can markedly slow or even completely block release of sialic acids by commonly used sialidases (1, 9, 38, 42–45) or by acid hydrolysis (9, 42, 46, 47). On the other hand, if stronger acidic conditions are used, significant destruction of the esters can occur (9, 20, 42, 48, 49). Also, many standard methods used in the structural analysis of oligosaccharides (including base hydrolysis during glycolipid purification, alkaline borohydride release of *O*-linked chains, hydrazinolysis, and methylation analysis) will cause destruction of *O*-acetyl esters. Even if modified sialic acids are released intact, their anomalous behavior in some colorimetric and chromatographic techniques can pose problems in analysis. Thus, conventional approaches to the study of sialic acids from biological sources can easily miss a significant amount of *O*-acetylation. In this study, we have used a set of techniques that permit the release, isolation, and analysis of side chain (7–8–9) mono- and di-*O*-acetylated sialic acids without substantial loss of the ester groups. It should be pointed out that even these approaches are inadequate for the analysis of 4-*O*-acetylated sialic acids and that their efficacy in studying some other kinds of sialic acids (*e.g.* tri-*O*-acetylated, methylated, or sulfated) is unknown.

The selective enrichment of *O*-acetylation on *N*-linked oligosaccharides we have reported here is striking and stands in contrast to the selective *O*-acetylation of α2–8-linked sialic acids in gangliosides in other systems (28, 50–54). In human melanoma cells, *O*-acetylation seems to be confined to gan-

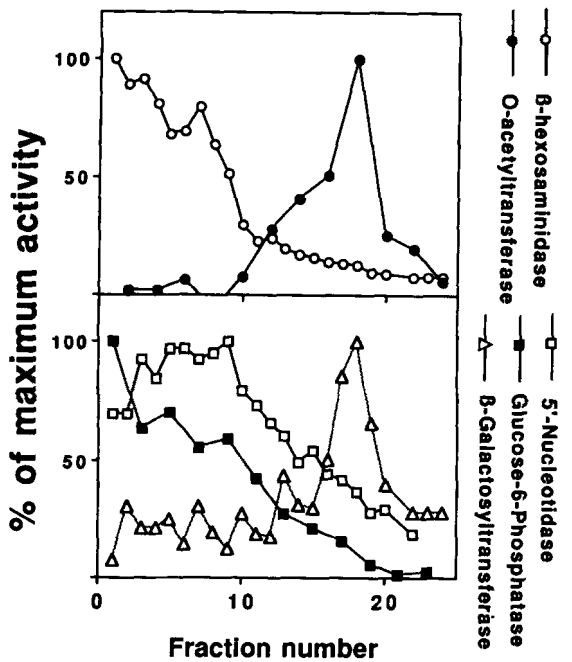

FIG. 5. **Sucrose density gradients of rat liver homogenates.** A postnuclear supernate of a rat liver homogenate prepared in 0.25 M sucrose, sodium maleate buffer, pH 6.5, was layered over a continuous sucrose density gradient (0.5–1.5 M) made in the same buffer and centrifuged for 100,000 × *g* for 18 h. Fractions were collected from the bottom with a glass capillary tube. The various enzymes were assayed exactly as described under "Experimental Procedures."

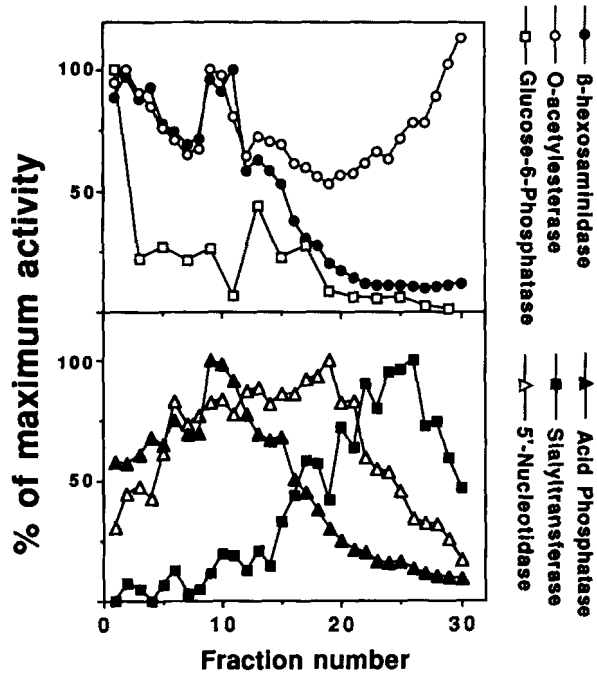

FIG. 6. **Sucrose density gradients of rat liver homogenates.** A postnuclear supernate of a rat liver homogenate prepared in 0.25 M sucrose, Tris/HCl, pH 7.4, was layered over continuous sucrose density gradient (0.5–1.5 M) made in the same buffer and centrifuged for 100,000 × *g* for 18 h. Fractions were collected from the bottom with a glass capillary tube. The various enzymes were assayed as described under "Experimental Procedures."

gliosides and is not found in glycoproteins from the same cell (17). Although technical limitations preclude a conclusive statement, it appears that most, if not all, of the *O*-acetylation of sialic acids in rat liver membranes is on *N*-linked oligosaccharides. Interestingly, the small amount of Neu5Gc present in these oligosaccharides does not appear to be *O*-acetylated to a detectable extent. Our most recent studies in this system even suggest that the *O*-acetylation might be selectively found only on α2–6-linked sialic acids and not on α2–3-linked residues.[2] Taken together, these data suggest that the *O*-acetyltransferase(s) of rat liver may be distinct from those of neuroectodermal cells (selective *O*-acetylation of α2–8-linked sialic acids of gangliosides) and submaxillary gland (selective *O*-acetylation of *O*-linked oligosaccharides?).

We had reported previously that when [*acetyl*-^{3}H]AcCoA is incubated with isolated intact rat liver Golgi-enriched vesicles, label is transferred to endogenous acceptor glycoproteins (5). This reaction may involve the transmembrane transfer of acetyl groups via an acetyl intermediate (6), similar to that reported for the glucosamine acetyltransferase of lysosomes (55). We had also reported the presence of an endogenous sialate:*O*-acetylesterase activity in Golgi-enriched fractions that could simultaneously remove the labeled *O*-acetyl groups from these endogenous acceptors (5). Addition of detergent abolished not only the transfer to the endogenous acceptors but also the esterase activity against them, suggesting that both activities were contained in the same membrane-limited compartments. The esterase activity could also be substantially diminished by adding the serine esterase inhibitor DFP, which can cross membranes (5). However, these prior studies only identified these activities and did not determine their major subcellular location.

To date, exogenous macromolecular acceptors have not proven useful in detecting the *O*-acetyltransferase activity after membrane solubilization. Thus, the rat liver Golgi sialate:*O*-acetyltransferase(s) function(s) very efficiently against endogenous acceptors in intact vesicles but cannot be detected after solubilization with detergents. This lability to solubilization may perhaps explain why more than 20 years after the first report of a sialate:*O*-acetyltransferase (56), no such enzymes have yet been purified from any source. We therefore studied the subcellular distribution of the *O*-acetyltransferase activity toward *endogenous* acceptors and found that this reaction is highly enriched in Golgi fractions. Although these data are not strictly quantitative, they do indicate that the components necessary for the *O*-acetylation reaction (the transferase and the endogenous acceptors) are markedly enriched in Golgi preparations. Since *O*-acetylation follows the transfer of sialic acids to oligosaccharides, the Golgi compartment where this takes place must be a very late one. It is also possible that the reaction takes place in an unknown post-Golgi compartment that tends to co-enrich well in Golgi preparations.

In striking contrast to the transferase, the membrane-associated 9-*O*-acetylesterase activity that we first identified in Golgi vesicles (5) was not highly enriched in this fraction. Rather, it co-fractionates and co-enriches best with lysosomal markers in studies using different techniques of subcellular fractionation. In keeping with this localization, the enzyme is largely released in a water-soluble form from membrane vesicles by repeated freeze-thaw or saponin permeabilization. In the accompanying paper (57), we report that antibodies against the purified esterase show staining in a lysosomal pattern in permeabilized fixed cells and that Triton WR-1339 treatment of rats causes a density shift expected for a lysosomal enzyme. Preliminary results with immunoelectron microscopy appear to confirm the lysosomal and late endosomal localization of this enzyme.[3] We are currently pursuing the

[3] C. Butor, G. Griffiths, N. Aronson, and A. Varki, submitted for publication.

TABLE V

Co-fractionation of sialate:O-acetylesterase activity with subcellular fractions from rat liver

Starting from a single batch of rat liver, the different types of subcellular fractions were prepared by separate techniques, as described under "Experimental Procedures." Each final fraction was assayed for sialate:O-acetylesterase activity and for -fold enrichment over the starting material of standard markers (sialyltransferase for Golgi, 5′-nucleotidase for plasma membranes, and acid phosphatase for lysosomes). The lysosomes were studied at the first hypotonic lysis step, since the esterase is soluble.

Fraction	Fold-purification of marker over post-nuclear supernate				
	5′-Nucleotidase	Glucose 6-phosphatase	Sialyltransferase	Acid phosphatase	9-O-Acetylesterase
Plasma membrane	10.35	1.3	17.45	1.2	−2.48
Golgi	6.33	4.38	41.04	2.98	2.26
Lysosomes	5.22	0.23	7.32	9.54	12.84

possibility that lysosomes might not always have an acidic interior, perhaps explaining the neutral pH optimum of the intralumenal (lysosomal) sialic acid-specific 9-O-acetylesterase.

It is now well established that O-acetyl esters at the 7-position of sialic acids can undergo migration to the 9-position, if the hydroxyl group at the latter position is free (9, 10). Single O-acetyl groups at the 8-position are practically nonexistent, presumably because they undergo extremely rapid migration to the 9-position (10). However, the rate of migration of 7-O-acetyl groups is markedly sensitive to pH changes in the physiological range, at body temperature. Thus pH values in the mildly acidic range (6.5 or less) are not associated with detectable migration at 37 °C. In contrast, the $t_{1/2}$ for O-acetyl migration occurring in free sialic acids was reported to be 4–8 h at pH 7.0 and 37 °C (10). Increase of pH into the mildly alkaline range is associated with significantly more rapid migration. The basolateral cell surface of the hepatocyte forms the bulk of its plasma membrane and is exposed to blood plasma, which has a tightly regulated pH in the range of 7.35–7.45. In contrast, the internal organelles of the cell that could have sialic acid residues tend to have more acidic pH values, ranging from 6 to 6.5 for the trans-Golgi to 6.0–4.0 for endosomes and lysosomes (58). We found that 9-O-acetylation was preferentially enriched in plasma membrane fractions (exposed *in vivo* to pH > 7), whereas 7-O-acetylation was enriched in lysosomal membranes (exposed predominantly to low pH, with a 9-O-acetylesterase present). In keeping with earlier biosynthetic studies that showed O-acetylation taking place on both the 7- and the 9-positions, the Golgi-enriched fractions contained both types in a ratio of about 3:5. For the purification of each type of subcellular fraction the best available protocol for enrichment was followed. It is recognized of course that no fraction is absolutely pure and that the markers themselves are only relatively specific (*e.g.* small amounts of β-hexosaminidase is normally found in the Golgi apparatus, and 5′-nucleotidase can be present in early endosomes). Regardless, the distribution of 7- and 9-O-acetylation is strikingly like that which one would predict emperically, based upon the pH values and enzymatic activities encountered by the different membranes, in the intact cell.

Based upon all these considerations, we offer a model that could explain the synthesis, distribution, and turnover of O-acetyl groups on the sialic acids of rat liver membranes (see Fig. 7). This general model could also be applicable to side chain O-acetylation in other systems. Note that it does not fully take into account the existence and turnover of di- and tri-O-acetylated sialic acids, nor the possibility that re-cycling of glycoconjugates could result in re-O-acetylation. Also, it assumes that *all* O-acetylation takes place in the Golgi apparatus, as currently defined. This remains somewhat difficult

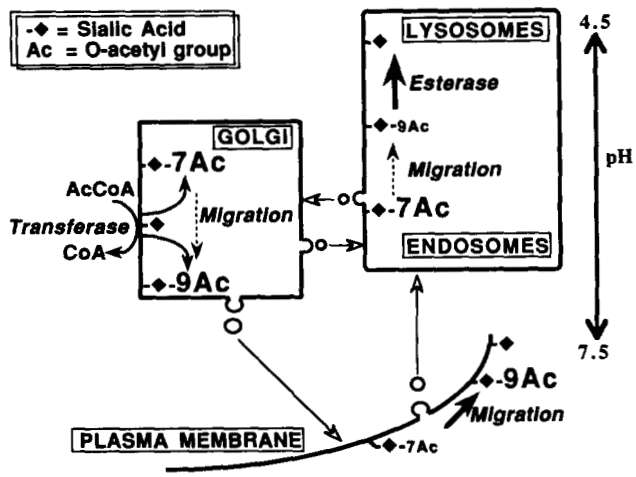

FIG. 7. **Probable pathways in the biosynthesis, migration, and turnover of 7- and 9-O-acetyl esters on glycosidically bound sialic acids in the rat liver.** This model takes into account data from this and previous studies. See text for discussion.

to reconcile with our inability to show *direct overlap between* sialylation and O-acetylation reactions in rat liver Golgi (5).[2] It is possible that O-acetylation actually takes place in an unknown compartment, beyond the *trans*-Golgi network where sialylation is felt to be predominantly located. This can only ultimately be proven when the O-acetyltransferase enzyme(s) are purified and characterized and appropriate probes for their direct detection are generated.

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