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Characterization of the Enzymatic 7-*O*-Acetylation of Sialic Acids and Evidence for Enzymatic *O*-Acetyl Migration from C-7 to C-9 in Bovine Submandibular Gland[1]

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Microsomes prepared from bovine submandibular glands incubated with radioactive AcCoA incorporated acid-insoluble radioactivity, which was dependent on time, and the concentrations of AcCoA and proteins, and was inhibited by CoA in a concentration-dependent manner. Under the conditions used, the apparent K_m for AcCoA was 1.63 μM with a V_{max} of 21.9 pmol/mg protein·min. The radioactivity incorporated was mainly due to the *O*-acetylation of glycosidically bound Neu5Ac. The primary attachment site of *O*-acetyl groups was exclusively the hydroxyl at C-7 of Neu5Ac, the presence of an AcCoA:Neu5Ac 7-*O*-acetyltransferase thus being demonstrated. After longer incubation 9-*O*-acetylated Neu5Ac also appeared, suggesting the migration of an ester group from C-7 to C-9. This isomerisation was inhibited by heat-inactivation of the microsomal protein, enzymatic isomerisation by a "migrase" thus being suggested. Data are presented which lead to the assumption that this 7-*O*-acetylation involves at least two reactions: the transport by a translocase of acetyl groups from AcCoA from the cytosol across the Golgi membrane, followed by the enzymatic transfer of these acetyl groups onto sialic acids in the Golgi lumen.

Key words: bovine submandibular gland, Golgi fraction, *O*-acetyl migration, sialate 7-*O*-acetyltransferase, sialic acid.

Sialic acids occur in microorganisms and higher animals as components of oligosaccharides, polysaccharides, glycoproteins, and glycolipids. They form a large family comprising about 40 derivatives of neuraminic acid (systematic name: 5-amino-3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranos-1-onic acid) (*1, 2*). Their involvement in numerous biological events, and in particular in the regulation of cellular and molecular recognition, is now well established (*1-3*).

Naturally occurring sialic acids can be *O*-acetylated at their 4-, 7-, 8-, and 9-hydroxyl groups, yielding mono- to tri-*O*-acetylated species. Rich sources for these sialic acids are the mucins from equine and bovine submandibular glands and human colonic epithelium (*1-3*). The *O*-acetylation results from the activity of at least two distinct enzymes: AcCoA:sialate 4-*O*-acetyltransferase [EC 2.3.1.44] and AcCoA:sialate 7(9)-*O*-acetyltransferase [EC 2.3.1.45] (*4-6*). The latter enzyme seems to be widespread, because 9-*O*-acetylated sialic acids have been found in bacteria and many animal species including man (*1-3*).

Interest in *O*-acetylated sialic acids has increased during the last years, since the presence of ester groups can be correlated with various biological and pathological phenomena. One of the best documented roles of *O*-acetylated sialic acids is hindrance of the degradation of glycoconjugates (*7*), since *O*-acetyl groups induce partial or total inhibition of the action of sialidases as well as of sialate pyruvate-lyases. They also play important roles in cellular communication and immunity, and can influence the antigenicity of bacterial polysaccharides (*8*). For example, the binding of sialoadhesin and CD22 to their ligands is inhibited by sialic acid *O*-acetylation (*9, 10*). *O*-Acetylated sialic acids are differentiation molecules on the surface of chicken erythrocytes (*11*), and are markers of murine CD4 T cells involved in the maturation and activation of lymphocytes (*12*). These *O*-acetyl groups are also involved in infectious diseases, playing a dual role in the binding of pathogens to host cells. Neu5,9Ac$_2$ was shown to serve as a receptor determinant for some viruses, which was first observed for influenza C virus (*13*), whereas for other pathogens it can prevent their adhesion. The best known

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Abbreviations: AcCoA, acetyl coenzyme A; AUS, *Arthrobacter ureafaciens* sialidase; BSA, bovine serum albumin; BSM, bovine submandibular gland mucin; *p*-CMB, *p*-chloromercuribenzoate; CMP-Neu5Ac, cytidine 5′-monophosphate-*N*-acetylneuraminic acid; CoA, coenzyme A; DEPC, diethylpyrocarbonate; DFP, diisopropylfluorophosphate; DMB, 1,2-diamino-4,5-methylenedioxybenzene; DTT, dithiothreitol; NBS, *N*-bromosuccinimide; Neu2en5Ac, *N*-acetyl-2-deoxy-2,3-didehydro-neuraminic acid; Neu5Ac, *N*-acetylneuraminic acid; Neu5,7Ac$_2$, *N*-acetyl-7-*O*-acetylneuraminic acid; Neu5,8Ac$_2$, *N*-acetyl-8-*O*-acetylneuraminic acid; Neu5,9Ac$_2$, *N*-acetyl-9-*O*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; NDVS, Newcastle Disease Virus sialidase; PK-buffer, 50 mM potassium phosphate buffer, pH 6.5, containing 50 mM KCl; PMSF, phenylmethylsulfonyl fluoride; TLC, thin-layer chromatography.

example of the latter effect is that the *O*-acetylation of sialic acids inhibits the binding of and thus the infection by influenza A and B viruses, which only recognize unmodified, terminal Neu5Ac residues (*14, 15*). Aberrant expression of *O*-acetylated groups accompanies physiological differentiation or malignant transformation of cells or tissues: for example, *O*-acetylation decreases in human colonic cancer cells (*16-19*) but increases on T-lymphocytes from patients with brain cancers and melanomas (*20*). For further examples concerning the biological impact of sialic acid *O*-acetylation see the reviews (*1-3, 21*).

So far, sialate 7(9)-*O*-acetyltransferase has mainly been studied in rat liver (*22-26*), and evidence has been presented that the *O*-acetylation of glycoconjugate-bound sialic acids in the Golgi apparatus is a complex mechanism involving both an AcCoA transporter and an intraluminal real *O*-acetyltransferase. We describe the activity of the corresponding enzyme in bovine submandibular gland, a tissue in which 7(9)-*O*-acetyltransferase activity was observed for the first time (*4, 5*). Because the cow is an animal known to synthesize in submandibular glands most kinds of *O*-acetylated sialic acids (*27, 28*), and in particular since over 80% of the sialic acid residues from the mucin of submandibular glands are *O*-acetylated (*1-3, 6*), we used fresh bovine submandibular glands as experimental material. Furthermore, since this easily accessible and relatively large organ produces a high amount of *O*-acetylated sialomucins, the corresponding enzymes might be very active or produced in large amounts.

On the other hand, while 4-*O*-acetyltransferase is known to be specific for the hydroxyl group at C-4 of the pyranose ring of neuraminic acid, the primary attachment site of the acetyl residue transferred by 7(9)-*O*-acetyltransferase remains a point of discussion. Since it is well established that an *O*-acetyl ester at the 7-position of sialic acids can migrate spontaneously to the 9-position of the glycerol side-chain (*29*), a hypothesis was forwarded, according to which this enzyme would effectively be a 7-*O*-acetyltransferase incorporating *O*-acetyl groups primarily at C-7 of sialic acids, followed by their migration to the primary hydroxyl group at C-9 and further transfer of acetyl residues to C-7, di- and tri-*O*-acetylated species thus being yielded (*30*). However, this hypothesis remained to be confirmed. Butor *et al.* (*25*) demonstrated that in rat liver the primary attachment site of acetyl groups may be regulated as a function of the pH in different cell compartments with preferential 9-*O*-acetylation in plasma membrane fractions, 7-*O*-acetylation in lysosomal membranes, and both types of *O*-acetylation in Golgi vesicles.

We demonstrate that in the case of bovine submandibular glands, which predominantly contain 7- and 9-*O*-acetylated sialic acids, the radioactive ester groups are primarily transferred to the C-7 hydroxyl group of sialic acid residues, probably followed by enzyme-catalyzed migration to C-9.

MATERIALS AND METHODS

Materials—[1-^{14}C]Ac-CoA [57 mCi/mmol (2.11 GBq/mmol)], [^{3}H]Ac-CoA [4.00 Ci/mmol (148 GBq/mmol)], and CMP-[4,5,6,7,8,9-^{14}C]Neu5Ac [286 mCi/mmol (106 GBq/mmol)] were purchased from Amersham (Amersham, UK). *Arthrobacter ureafaciens* (AUS) sialidase was purchased from Calbiochem (La Jolla, USA), and Newcastle Disease Virus (NDVS) sialidase as well as Neu2en5Ac from Boehringer Mannheim (Mannheim, Germany). Ficoll was from Pharmacia Biotech (Uppsala, Sweden), Dowex 50W-X8 (20-50 mesh, hydrogen form), and AG 1-X8 (200-400 mesh, formate form) were from Bio-Rad Laboratories (Hercules, USA), and fetuin was obtained from Sigma Chem. (St. Louis, USA).

Subcellular Fractionation of Bovine Submandibular Glands—Bovine submandibular glands were acquired from a slaughterhouse immediately after the death of the animals and kept cooled on ice until use. All the following procedures were carried out at 4°C unless otherwise described. After removing fat and connective tissue, the glands were hashed with a meat grinder. Three hundred grams of the triturated glands was washed in 4 liters of 10 mM Tris/HCl buffer, pH 7.2, and then filtered through a sieve to remove the bulk of mucin. The subcellular fractionation was performed according to the method described by Schauer *et al.* (*31*) with slight modifications: After the addition of 100 ml 0.1 M Tris/HCl buffer, pH 7.2, the ground, washed tissue material was homogenized in an ice bath using an Ultraturrax at 24,000 rpm for 5 sets of 30 s each with a 30 s pause between each set. One-third of the resultant "crude homogenate" was mixed with 300 ml of 3% Ficoll in Tris buffer and then rehomogenized under the same conditions. This homogenate was centrifuged for 1 h at 100,000×*g*. The pellet was resuspended in 150 ml of 1.5% Ficoll in Tris buffer and again homogenized using a Potter homogenizer with 10 strokes at the highest setting. Centrifugation at 1,200×*g* for 15 min gave a pellet consisting of two layers. After discarding the lipids, the upper, soft layer of the pellet was carefully resuspended in the supernatant without disturbing the compact, lower layer. The membrane suspension, which was almost free of nuclear and connective tissue material (*31*), was fractionated on a discontinuous Ficoll density gradient. Twenty-two percent Ficoll (5.4 ml) in Tris buffer was transferred to a 38 ml Beckman SW 28 tube and then overlaid successively with 5.4 ml each of 19, 17, 15, and 9% Ficoll buffer, and finally with 8 ml homogenate in 1.5% Ficoll. The gradient mixtures were centrifuged for 2 h at 64,000×*g* and the six different fractions obtained were carefully harvested in a minimum volume with a Pasteur pipette, diluted 10-fold in 0.1 M Tris/HCl buffer, pH 7.2, and then centrifuged at 100,000×*g* for 30 min. The resulting pellets were frozen and stored at −80°C until use.

Preparation of Microsomal Membranes—The remaining two-thirds of the above "crude homogenate" was mixed with 600 ml 0.1 M Tris/HCl buffer, pH 7.2, homogenized again under the same conditions as before and then centrifuged for 1 h at 100,000×*g*. The pellet was resuspended in 150 ml of Tris buffer and then homogenized in a Potter homogenizer with 10 strokes at the highest setting. The homogenate was then centrifuged for 1 h at 125,000×*g*, and the upper, yellowish compact microsomal layer of the pellet was distributed into Eppendorf caps and frozen at −80°C until use. This preparation will be termed "total microsomes" from hereon.

O-Acetyltransferase Assay Involving Endogenous Acceptors—Three hundred to 600 μg of membrane proteins (fractionated membranes or total microsomes) was routinely incubated with [1-^{14}C]Ac-CoA or [^{3}H]Ac-CoA (each

adjusted to 16.9 μM, 0.1 μCi, with unlabeled AcCoA) in 250-500 μl PK-buffer (50 mM potassium phosphate, pH 6.5, 50 mM KCl) at 22°C for 5-20 min, in an ultracentrifuge tube, according to Diaz *et al.* (*23*) with slight modifications. The time, temperature, and protein and AcCoA concentrations as well as the additions of various inhibitors and detergents are indicated in tables and figures under "RESULTS." In order to inhibit sialate-*O*-acetylesterase activity possibly present, in some experiments the membranes were preincubated for 15 min with 1 mM DFP at 4 or 22°C. The incubation was stopped by diluting the unused AcCoA with 10 ml ice-cold PK-buffer. After centrifugation at 100,000 × *g* for 1 h, the surface of the pellet was gently washed twice with 4 ml ice-cold buffer. The pellet was then resuspended by sonication in 250 μl ice-cold water and transferred to an Eppendorf ultracentrifuge cap. Eight percent perchloric acid (250 μl) was used to rinse the first ultracentrifuge tube and then transferred to the cap. After mixing, the membrane-bound glycoproteins were left for 1 h on ice to precipitate. After centrifugation at 21,000 × *g* for 10 min in a Beckman TL-100 Tabletop ultracentrifuge, 80% of the supernatant was counted by liquid scintillation. The rest was discarded and the surface of the pellet was rinsed gently with 250 μl 4% perchloric acid, of which 80% was also counted. After correction, both acidic fractions represented the "acid-soluble radioactivity." The pellet was finally dissolved in 350 μl of 0.1 N NaOH for 15 min at 80°C. After mixing, 175 μl of ice-cold 0.2 N HCl was added and "acid-insoluble radioactivity" was measured by liquid scintillation. The results were calculated as 100% and expressed as average values in pmol acetate transferred. The protein concentration of membrane homogenates were determined by the modified Lowry method (*32*) with bovine serum albumin as the standard. Experiments were carried out at least in duplicate.

Sialyltransferase Assay—The membrane fractions were rehomogenized in 0.1 M cacodylate buffer, pH 6.5, containing 0.1% BSA, 0.2 M galactose, 1 mM Neu2en5Ac, and 1.67% Triton X-100. They were then incubated with CMP-[^{14}C]Neu5Ac (23.92 μM, 0.1 μCi) and 50 μl asialo-fetuin (4 mg/ml H_2O) in a final volume of 255 μl for 6 h at 37°C (*33*). The reaction was stopped by cooling on ice. After short centrifugation at 14,000 × *g*, 12 μl of the supernatant was loaded onto a cellulose TLC plate, and run overnight in 95% ethanol/1 M ammonium acetate, pH 7.5 (7:3 by volume), and then sialyl transfer was quantitated by radio-TLC (*34*) using an automatic TLC-Linear Analyser LB 284 (Berthold, Wildbad, Germany). The results were expressed as average values in nmol of Neu5Ac residues transferred per mg of protein.

Enzymatic Release of Sialic Acids—After incubation of Golgi membranes or the fraction of total microsomes with radioactive AcCoA as above described, the washed 100,000 × *g* pellet was resuspended in the ultracentrifuge tube by sonication in 250 μl of 0.1 M sodium acetate buffer, pH 5.5, containing 0.5% Triton X-100, and then transferred to an ultracentrifuge cap. After rinsing of the first tube with one volume of acetate buffer and adding the washing to the sonicate, sialic acids were enzymatically released by incubation at 37°C for 1-3 h with 20-40 mU of AUS or 20 mU of NDVS. Acid-soluble, also including sialidase-susceptible radioactivity, and acid-insoluble radioactivity were determined as described above and compared to those in controls without sialidase.

Saponification of Neo-O-Acetylated Sialic Acids—After incubation of microsomes for 20 min with [^{3}H]Ac-CoA, the washed 100,000 × *g* pellet was resuspended by sonication in 250 μl ice-cold water as described above. Sialic acids were de-*O*-acetylated by incubating the samples for 30 min at 37°C in 0.1 N NaOH with or without 0.3% Triton X-100 in a final volume of 500 μl. Before precipitation of the acid-insoluble radioactivity with 4% perchloric acid, the incubates were neutralized with HCl. Controls were incubated for 30 min at 37°C in 0.1 N NaCl with or without Triton X-100. Acid-soluble and acid-insoluble radioactivity was determined as described above.

Chemical Release and Analysis of Neo-O-Acetylated Sialic Acids—After incubation with radioactive AcCoA, both the preformed and neo-*O*-acetylated sialic acids were released from endogenous substrates by incubation of the washed 100,000 × *g* pellet after sonication for 4 h at 80°C in 2 M propionic acid, pH 2.3 (*35*), cooled on ice and then briefly centrifuged. The supernatants were lyophilized to eliminate the acid. Sialic acids were then purified by sequential ion-exchange chromatography: the lyophilizates were dissolved in 100 μl water, and then loaded onto a 500 μl Dowex 50W-X8 column (20-50 mesh, H^+ form, in water) and eluted with 10 ml water. The pH of the eluents was controlled and if necessary adjusted to 5 with diluted fresh ammonia. The eluents were directly loaded onto a 500 μl AG 1-X8 column (200-400 mesh, $HCOO^-$ form, in water). After washing with 8 ml water, sialic acid residues were eluted with 10 ml 1.5 M formic acid. All these purification steps were carried out at 4°C to minimize hydrolysis and the rate of migration of *O*-acetyl groups. After the elimination of formic acid by two lyophilizations, the purified sialic acids were analyzed by radio-TLC on cellulose sheets, pre-run twice, using the solvent system of *n*-butanol/*n*-propanol/0.1 M HCl (1:2:1 by volume). Standard sialic acids used as references were isolated from bovine submandibular glands and visualized using the orcinol/Fe^{3+}/HCl spray reagent (*36*).

HPLC Analysis of Endogenous Sialic Acids—Endogenous sialic acids chemically released from bovine submandibular gland mucin or the total Golgi membrane fraction were analyzed by a modification of the method by Hara *et al.* (*37*). After mild acidic hydrolysis with propionic acid as described above, the samples were cooled on ice and then briefly centrifuged at 14,000 × *g* in a table-centrifuge. Ten microliters of each supernatant was incubated for 1 h at 56°C in the dark with 50 μl of a DMB solution (15.75 mg DMB dissolved in 5 ml water, 680 μl 2-mercaptoethanol, 39 μl 37% $NaHSO_3$, and water to 10 ml). After derivatization, the samples were cooled on ice and then 20 μl was analyzed by HPLC on a reversed-phase column (RP18, 250 × 4 mm, particle size 5 μm; from Merck, Darmstadt, Germany). The mobile phase was a mixture of acetonitrile-methanol-water (9:7:84, by volume), the flow rate was 1 ml/min, the emission and excitation wavelengths were 448 and 373 nm, respectively, and the fluorescence detector was from Applied Biosystems (New Jersey, USA).

"Migrase" Assay—After incubation of the Golgi-enriched fractions for 10 min with [1-^{14}C]Ac-CoA, 10 μl of 60 mM DEPC in PK-buffer was added or the samples were heated for 5 min at 95°C and then cooled for 2 min on ice. In

both cases, the samples were left for a further 20 min at 22°C after this treatment. The ratios of radiolabeled Neu5,7Ac$_2$ and Neu5,9Ac$_2$ residues were determined by radio-TLC after hydrolysis and purification of neo-*O*-acetylated sialic acids as described above and compared to controls after 10 or 30 min incubation.

RESULTS AND DISCUSSION

Analysis of Endogenous Sialic Acids—Like other enzymes involved in anabolism, and in particular in the terminal modification of glycoconjugates, sialate *O*-acetyltransferases, which transfer *O*-acetyl esters to glycosidically bound sialic acids, appear to be located in microsomal fractions from rat liver and bovine submandibular gland (*22, 31*). Since we were mainly interested in the primary site of incorporation of *O*-acetyl groups into sialic acids, it was necessary first to determine the nature of potential endogenous substrates for this enzyme, *i.e.* the kind of sialic acids at the subcellular site of enzyme localization. However, no method is known which enables exact quantitative and qualitative determination of *O*-acetylated sialic acids in biological materials, due to incomplete release from glycosidic linkages as well as loss and migration of *O*-acetyl groups during the isolation and purification of the liberated sialic acids (*38*). We therefore compared the method suggested earlier (*36*) for the release of *O*-acetylated sialic acids involving formic acid, pH 2, for 1 h at 70°C with a newly proposed method involving 2 M propionic acid, pH 2.3, at 80°C for 1 to 4 h (*35*). To avoid further loss of ester residues, we omitted the purification steps for the released sialic acids, which involve sequential ion-exchange chromatographies (*36*), and analyzed the sialic acids directly after hydrolysis by fluorometric HPLC (*37*).

The results of the two mild acid hydrolysis procedures for bovine submandibular gland mucin are compared in Table I, which demonstrates that after 4 h more sialic acids were released with propionic acid than with formic acid after 1 h (78% compared to 38%). Furthermore, a higher amount of *O*-acetylated sialic acids was released with propionic acid than with formic acid, and also involved less de-*O*-acetylation and a relatively higher proportion of 7-*O*-acetylated sialic acids when compared with 9-*O*-acetylated derivatives, implying less migration of ester groups from C-7 to C-9. In addition, the proportions of di- and tri-*O*-acetylated sialic acids were higher with propionic acid, even after prolonged hydrolysis and at the higher temperature, than observed with formic acid. One of the reasons for the advantage of propionic acid over formic acid may be the fact that the former evaporates azeotropically with water and thus does not concentrate on lyophilization in contrast to formic acid, which tends to be concentrated and thus may damage the sensitive *O*-acetylated sialic acids. We therefore used propionic acid for the release of sialic acids in the following enzymatic studies.

In Golgi-enriched membranes isolated from bovine submandibular glands, a total amount of 25–30 μg sialic acids per mg of protein was found. The predominant sialic acids were unsubstituted Neu5Ac and Neu5Gc, and among the *O*-acetylated species Neu5,9Ac$_2$ was predominant (Table II).

Incorporation of Radioactivity from Acetyl-Coenzyme A into Microsomes—The properties of the membrane-bound *O*-acetyltransferase activity as to endogenous substrates were determined according to classical methods using membranes of microsomal fractions from bovine submandibular glands as the enzyme source. As shown in Fig. 1, the total microsomal fraction incorporated acid-soluble and acid-insoluble radioactivity in a manner dependent on the time (Fig. 1A), as well as on the concentrations of AcCoA (Fig. 1B) and protein (Fig. 1C). Figure 1B shows the saturation of the acid-insoluble incorporation at about 10 μM AcCoA. That this value seems to be high may be explained by the fact that the microsomes contained a relatively large quantity of endogenous substrates. The dependence on the protein concentration is expressed in Fig. 1D as specific enzyme activity, which shows a decrease in the acid-insoluble incorporation of radioactivity at concentrations higher than 1.6 mg protein/ml under the experimental conditions used. Since the AcCoA concentration (about 17 μM) was not saturating, the data indicate that beyond 1.6 mg protein/ml this decrease was not due to a lack of AcCoA but that the quantity of mucins attached to the microsomes, being then too great in the incubate, may have inhibited the enzyme activity by increasing the viscosity.

TABLE I. **Qualitative and quantitative comparison by fluorimetric analyses of the total amounts and individual sialic acids released from mucins isolated from bovine submandibular glands by formic acid and propionic acid, respectively.** The total amount (27 μg sialic acid/100 μg dry mucin taken as 100%) of sialic acids was determined by means of the orcinol/Fe^{3+}/HCl assay (*36*).

Hydrolysis	Formic acid pH 2, 70°C	Propionic acid 2 M, pH 2.3, 80°C			
Incubation time	1 h	1 h	2 h	3 h	4 h
% released from total sialic acids	37.72	39.86	51.96	66.90	78.29
Composition of released sialic acid fraction (%)					
Neu5Gc	34.28	34.26	31.91	32.78	32.98
Neu5Ac	45.01	41.66	40.67	37.46	31.26
Neu5,7Ac$_2$	1.98	2.10	2.69	3.28	3.56
Neu5Gc9Ac	3.76	2.18	2.27	1.99	1.61
Unknown peak[a]	3.34	5.55	6.96	9.45	14.88
Neu5,9Ac$_2$	9.10	10.48	11.12	10.06	9.89
Di-*O*-acetylated	0.68	1.31	1.04	1.08	1.52
Tri-*O*-acetylated	1.85	2.46	3.34	3.90	4.30

[a]has the same R_t as 4,8-anhydro-Neu5Ac.

TABLE II. **Fluorimetric determination of the nature of endogenous, Golgi-membrane-bound sialic acid residues released on propionic acid hydrolysis.** The results are expressed as the average values for 5 analyses. The amount of total sialic acids released per mg protein was 27.5±2.5 μg.

Sialic acid	Percent of total fraction
Neu5Gc	23.18±7.18
Neu7Ac5Gc[a]	0.47
Neu9Ac5Gc	1.85±0.54
Neu5Ac	39.40±6.55
Neu5,7Ac$_2$	2.09±2.03
Neu5,8Ac$_2$[b]	5.94±5.68
Neu5,9Ac$_2$	11.18±2.07
Unknown peak[c]	5.98±4.75
Di-*O*-acetylated	5.37±4.98
Tri-*O*-acetylated	4.53±3.22

[a]was observed only once. [b]no standard available, by exclusion only. [c]has the same R_t as 4,8-anhydro-Neu5Ac.

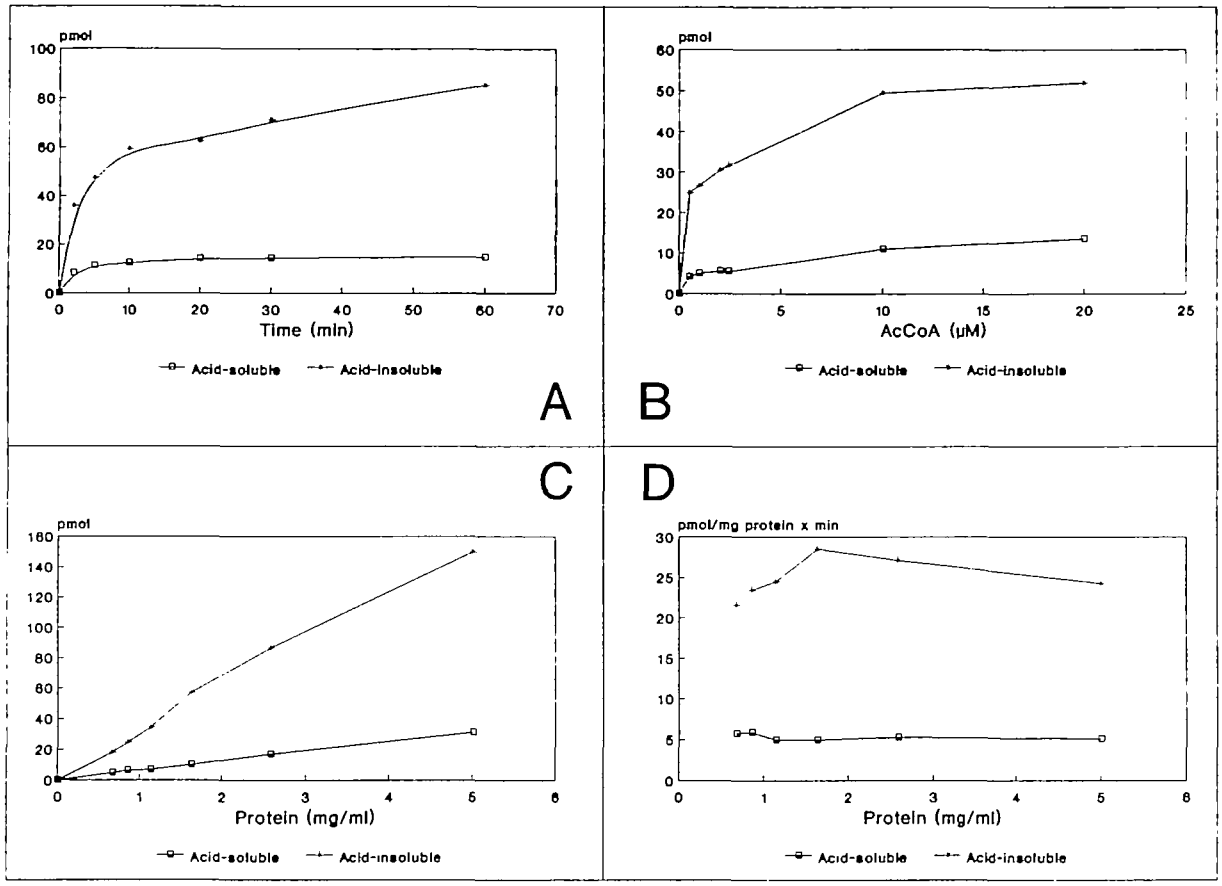

Fig. 1. **Incorporation of radioactivity from [1-^{14}C]Ac-CoA into total microsomal membranes from bovine submandibular glands depending on various parameters.** The conditions are given under "MATERIALS AND METHODS." The incubation times for panels B, C, and D were 5 min. The quantities of acetate transferred are indicated in pmol.

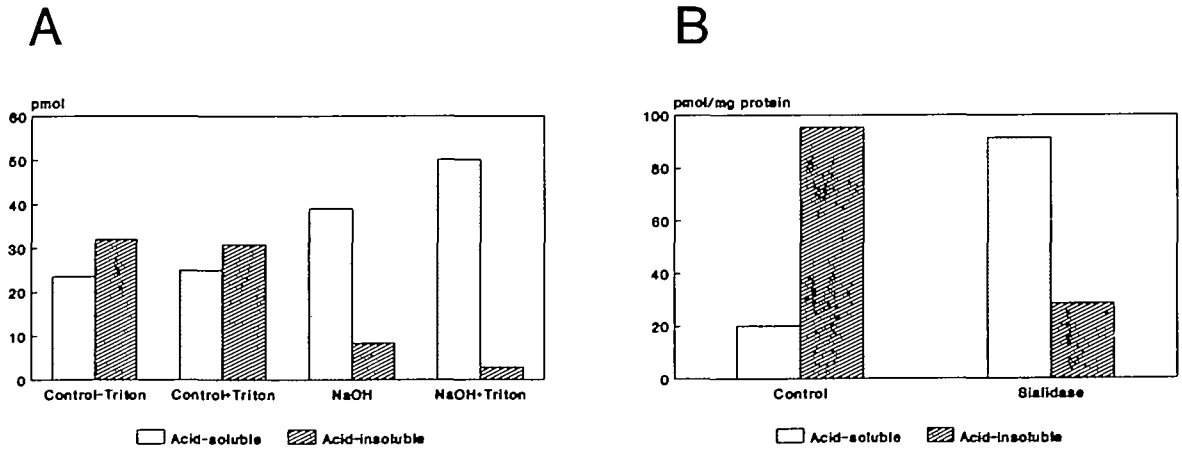

Fig. 2. **Saponification (A) and *Arthrobacter ureafaciens* sialidase treatment (B) of the radioactivity incorporated into total microsomes.** The conditions are given under "MATERIALS AND METHODS." The results of the incubation of microsomes with [^{3}H]AcCoA for 20 min are given as the averages for 6 experiments.

The acid-soluble component was almost exclusively free acetate, thus confirming the results of Varki and Diaz (22), whereas the acid-insoluble materials, which represented the larger part of the radioactivity incorporated into the total microsome fraction (Fig. 2), consisted mainly of neo-*O*-acetylated sialic acids. Figure 2A shows that saponification of radioactive samples greatly decreased the acid-insoluble radioactivity, whereas saponification carried out in the presence of 0.3% Triton X-100 almost completely abolished the acid-insoluble radioactivity. This demonstrates that the acid-insoluble radioactivity is the result of sialic acid *O*-acetylation in the microsomes. Correspond-

ingly, as shown in Fig. 2B, most (82% *versus* 24%) of the acid-insoluble radioactivity was removed from the microsomes and found in the acid-soluble fraction after sialidase treatment. Since AUS only releases non-*O*-acetylated and mono-*O*-acetylated sialic acids from their glycosidic bonds, leaving the oligo-*O*-acetylated species at glycosidic linkages (*37*), it can be concluded that the acid-insoluble radioactivity incorporated into microsomes corresponded mainly to mono-*O*-acetylation of sialic acids under the experimental conditions used.

Subcellular Localization of Enzyme Activity—Since the *O*-acetylation of sialylated glycoconjugates occurs after the action of sialyltransferases, which are located in the *trans*-Golgi network (*39-41*), the 7(9)-*O*-acetyltransferase activity was expected to be in this compartment. In order to confirm this hypothesis in the bovine system and also to localize the *O*-acetyltransferase, we fractionated microsomal membranes from fresh bovine submandibular glands on Ficoll discontinuous density gradients, and then compared both *O*-acetyltransferase and sialyltransferase activities in the different membrane fractions obtained using radioactive AcCoA and CMP-Neu5Ac.

Bovine submandibular glands are dense and rigid tissues, producing and containing very large amounts of viscous mucin. Because of these properties, homogenization and fractionation under classical conditions used for smoother tissues like liver are particularly difficult. For this reason we used the method described earlier for the same material (*31*), although with some modifications. However, the freezing and thawing steps of the original method caused partial loss of the *O*-acetyltransferase activity in our experiments (data not shown). In order to remove the majority of the viscous mucin, the glands were minced after the removal of fat and connective tissue, extensively washed and freed from mucin by decantation in large volumes of buffer, and finally passed through a sieve prior to the homogenization steps.

The different membrane fractions (F1-F6) in Fig. 3 were assayed for *O*-acetyltransferase activity. It was most pronounced in fractions F3 and F4, the highest specific activity being in fraction F3 (Fig. 4A). This allowed us to confirm the hypothesis forwarded above and to localize the highest *O*-acetyltransferase activity together with the highest sialyltransferase activity in the Golgi-enriched fraction (F3) (Fig. 4B).

Optimization of the O-Acetyltransferase Assay—Prior to determination of the optimal parameters for the *O*-acetyltransferase assay with bovine submandibular glands, the membranes were pre-incubated with DFP at 4 or at 22°C in order to inhibit esterase activity possibly present, which may interfere with the *O*-acetylation of sialic acids. In the presence and absence of DFP, the total microsomes showed acid-insoluble incorporation of the radioactivity from AcCoA of about 91 and 88% of the control levels, respectively, and the Golgi-enriched fractions showed levels of about 92 and 87%. This indicates that no significant endogenous esterase activity was present, which could induce de-*O*-acetylation of radioactive neo-*O*-acetylated sialic acids. We therefore omitted DFP in further experiments. Similarly, pre-incubation for 15 min with 1 mM PMSF, a protease inhibitor, or incubation with 1 mM Neu2en5Ac, a sialidase inhibitor, had no effect on the acid-insoluble incorporation of radioactivity.

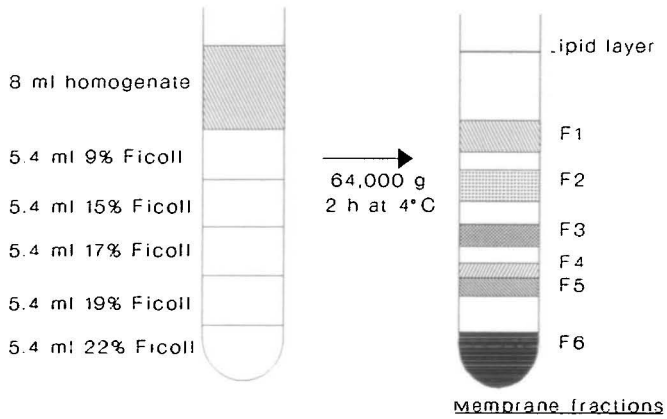

Fig. 3. **Fractionation of a microsomal preparation from bovine submandibular glands on a Ficoll discontinuous density gradient.** The membrane fractions are denoted as F1-F6. The method is based on the modified procedure described in Ref. *31*. For details, see "MATERIALS AND METHODS."

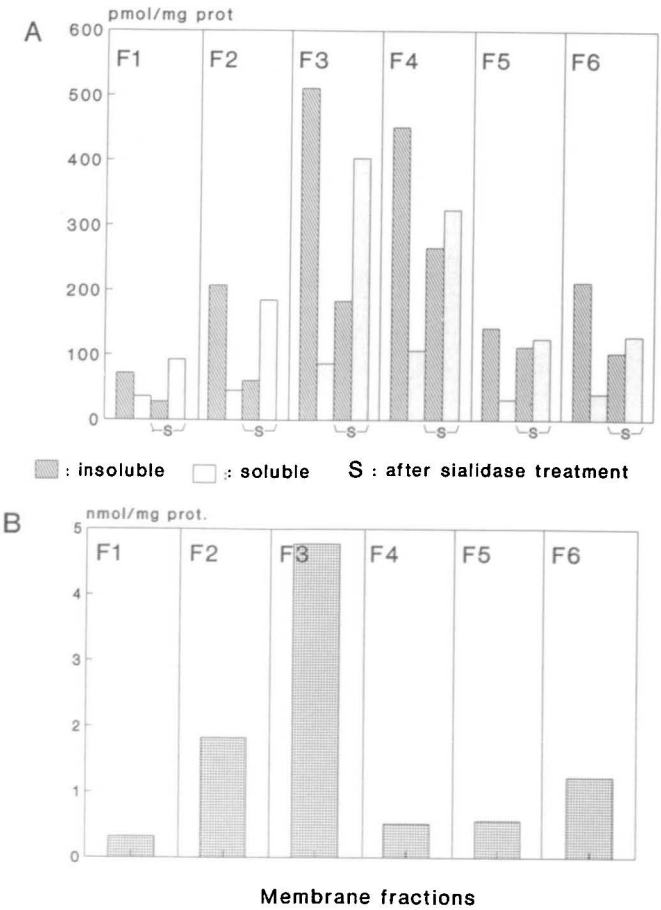

Fig. 4. **Sialate:*O*-acetyltransferase (A) and sialyltransferase (B) activities in subcellular membrane fractions (see Fig. 3) with endogenous acceptors.** Further details of the protocols used are given under "MATERIALS AND METHODS." The incubation time was 15 min for the *O*-acetyltransferase and 6 h for the sialyltransferase assays. The enzymatic *O*-acetylation was measured as the amount (pmol acetate) of acid-insoluble radioactivity formed per mg protein and was controlled by (partial) solubilization after 1 h treatment with *Arthrobacter ureafaciens* sialidase.

Under the conditions used, the apparent K_m of the *O*-acetyltransferase activity in the Golgi-enriched fraction for AcCoA was 1.6 μM with a V_{max} of 21.9 pmol/min·mg protein. The enzyme does not require divalent cations for optimal activity, since in the presence of EDTA, Ca^{2+}, Mn^{2+}, Mg^{2+}, and Fe^{2+} at 5 mM, transfer rates of insoluble radioactivity of 105, 102, 89, 104, and 97%, respectively, were found, compared to the control assay carried out in the absence of these substances.

Determination of the Nature of Neo-O-Acetylated Sialic Acids—The thin-layer radiochromatogram in Fig. 5A shows that the neo-*O*-acetylated sialic acids purified from endogenous substrates after 10 min incubation of Golgi-enriched fractions with [1-^{14}C]Ac-CoA had incorporated radioactive ester groups exclusively at the 7-hydroxyl position of the glycerol side chain of glycosidically bound Neu5Ac residues (peak I). After 30 min incubation (Fig. 5B), however, peak I was lower in favour of a new peak (peak II) that co-migrated with the sialic acid standard, Neu5,9Ac$_2$. This suggests that the 7-*O*-acetyl group can migrate to the free 9-position of a sialic acid during the longer incubation. It is therefore concluded that the *O*-acetylation of sialic acids in bovine submandibular glands is the result of the activity of an AcCoA:Neu5Ac 7-*O*-acetyltransferase, different from what has been observed in rat liver (*23*) and CHO cells (*42*), in which *O*-acetyl groups have been described to be primarily attached to positions 7 and 9, or to position 9 only. This would also be correlated to a mechanism of enzymatic *O*-acetylation and de-*O*-acetylation of sialic acids proposed earlier (*30*), according to which the primary incorporation site of *O*-acetyl groups would be C-7 of sialic acids, followed by non-enzymatic intramolecular migration of the ester group from C-7 to C-9, yielding di- and tri-*O*-acetylated species after further transfer of *O*-acetyl groups to C-7. Special studies are required to determine whether the *O*-acetyl group migrates directly to C-9 or *via* C-8. So far, no authentic Neu5,8Ac$_2$ is available and its existence in bovine submandibular gland mucin has not been unequivocally demonstrated (*28*). The fact, however, that peak II in Fig. 5, comigrating with reference Neu5,9Ac$_2$, is relatively broad may be explained by the possible presence of the intermediate, Neu5,8Ac2.

The observation that Neu5,7Ac$_2$ and Neu5,9Ac$_2$ (and possibly Neu5,8Ac$_2$) are the only radioactive compounds detectable after 30 min incubation, *i.e.* there is no *O*-acetylated Neu5Gc, although unsubstituted Neu5Gc and *O*-acetylated derivatives were also present in the fraction of endogenous sialic acids (about 25% of total sialic acids, see Table II), is probably due to the fact that the rates of *O*-acetylation of Neu5Gc residues are too low to be detected with the methods used. The ratios of Neu5Ac and Neu5Gc on the one hand and their respective 9-*O*-acetylated forms on the other, shown in Table II, being 1.7 *versus* 1 and 6 *versus* 1, respectively, may indicate that Neu5Gc is a poorer substrate than Neu5Ac for *O*-acetylation. Radioactive oligo-*O*-acetylated sialic acids were not found either, which may depend on the kinetics of the migration reaction and on the instability of the enzyme.

For determination of the nature of the glycosidic linkage of neo-*O*-acetylated sialic acids, two sialidases known to be

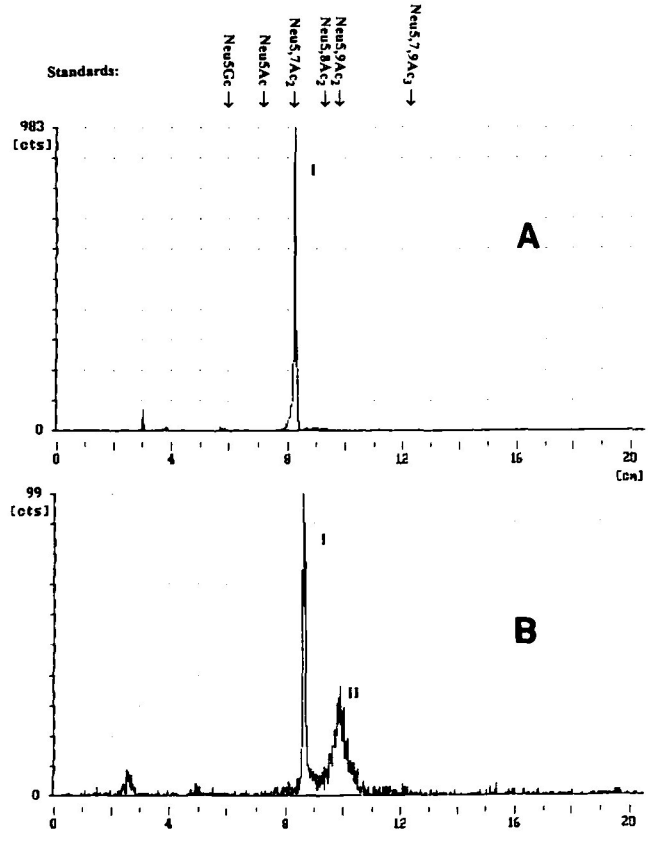

Fig. 5. **Radio-thinlayer chromatography on cellulose of sialic acids neo-*O*-acetylated in Golgi-fractions of bovine submandibular glands.** Membranes were incubated for either 10 min (A) or 30 min (B) with [1-^{14}C]Ac-CoA. I, Neu5,7Ac$_2$; II, Neu5,9Ac$_2$. Sialic acid standards were prepared from bovine submandibular gland mucin. The start and solvent front were at 3 and 20 cm, respectively. For further experimental details, see "MATERIALS AND METHODS."

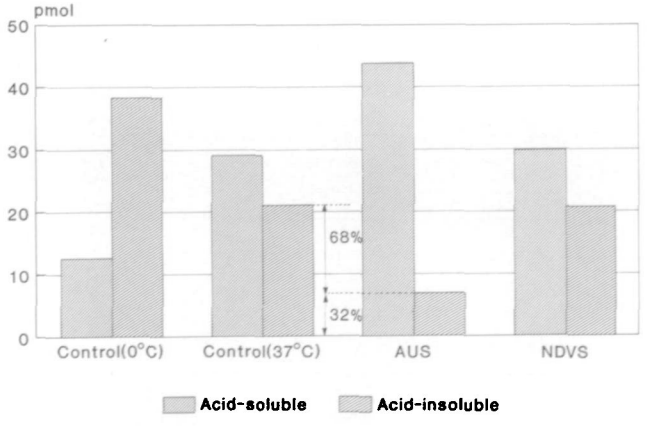

Fig. 6. **Determination of the anomeric linkage of neo-*O*-acetylated, endogenous sialic acids using different sialidases.** Microsomes were incubated for 20 min with [^{3}H]Ac-CoA prior to sialidase treatment. Control, 0°C: after *O*-acetyl transfer, the sample was left for 3 h on ice in sialidase buffer. Control, 37°C: the sample was incubated for 3 h at 37°C in sialidase buffer. AUS and NDVS: incubation with one of the two sialidases for 3 h at 37°C, followed by determination of the acid-soluble and residual acid-insoluble radioactivity (corresponding to pmol acetate). For the exact conditions, see "MATERIALS AND METHODS."

able to hydrolyze *O*-acetylated sialic acids (*5, 7*) were used. As shown in Fig. 6, AUS, that hydrolyzes α2,6-linked sialic acids about twofold faster than α2,3- and α2,8-linked sialic acids (*43*), released 68% of the acid-insoluble radioactivity compared to the control assay. On the other hand, NDVS, that exclusively hydrolyzes α2,3- and α2,8-linked sialic acids (*7, 44*), had no such effect. This indicates that the neo-*O*-acetylated sialic acids in bovine submandibular gland mucin are α2,6-linked, which correlates to the *O*-glycan structures of BSM described in the literature (*45*). Selective *O*-acetylation of α2,6-linked sialic acids was also reported in CHO cells (*42*), however, in contrast to bovine submandibular glands, it occurred on *N*-linked oligosaccharides. The fact that the ratios of acid-insoluble and acid-soluble radioactivities are so different in the two control assays in Fig. 6 is due to the long incubation period (3 h) at different temperatures and at pH 5.5, which may have led to the saponification of some *O*-acetyl residues, as well as to the hydrolysis of some sialic acid linkages by endogenous sialidases in the presence of Triton X-100.

Evidence for Enzymatic Migration of O-Acetyl Groups from C-7 to C-9—In order to determine whether the observed migration of *O*-acetyl groups from the C-7 to the C-9-position of Neu5Ac is due to physical isomerisation or to enzymatic activity, Golgi-enriched fractions were first incubated for 10 min at 22°C with radioactive AcCoA to obtain radiolabelled Neu5,7Ac$_2$ residues (see Fig. 7A) and then treated for 5 min at 95°C in order to stop the enzyme action. After cooling on ice, the incubates were left for a further 20 min at 22°C to allow the migration of acetyl groups. As shown in Fig. 7D, no 9-*O*-acetylated sialic acid species appeared after heat treatment, in contrast to the control assays without heating (Fig. 7B), in which the migration of about 75% of the *O*-acetyl groups from C-7 to C-9 occurred. This suggests that the isomerisation of these ester groups is caused by an enzymatic reaction and not by a physical phenomenon.

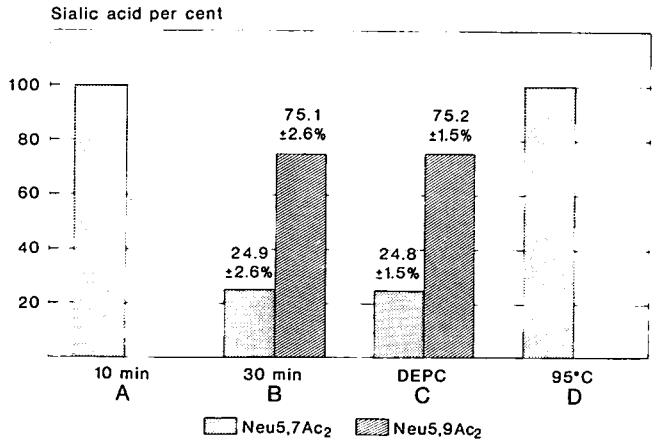

Fig. 7. **Inhibition of the migration of *O*-acetyl groups from C-7 to C-9 of sialic acids.** After the incubation of Golgi-fractions for 10 min with [1-^{14}C]Ac-CoA (A), DEPC was added (C) or the samples were treated at 95°C (D) and then left for a further 20 min at 22°C. The ratios of radiolabelled Neu5,7Ac$_2$ and Neu5,9Ac$_2$ residues were compared to controls after 10 min (A) or 30 min incubation (B). It can be seen that no isomerisation, *i.e.* Neu5,9Ac$_2$ formation, occurred in the case of heat inactivation (D), in contrast to the control assay (B) or the DEPC-treated sample (C) incubated for 30 min.

In a second experiment, after incubation for 10 min at 22°C with radioactive AcCoA, the samples were treated with DEPC, which inhibits 7-*O*-acetyltransferase (see below and Table III), and then left for a further 20 min at 22°C. Under these conditions, the same distribution of the radioactivity between Neu5,7Ac$_2$ and Neu5,9Ac$_2$ was observed as after 30 min incubation without the inhibitor (Fig. 7, B and C). This experiment also indicates the possibility of the existence of an enzyme responsible for *O*-acetyl migration, which cannot be inhibited by DEPC and is not associated with the 7-*O*-acetyltransferase activity.

In conclusion, the migration of the ester group to the C-9 position after its primary binding to the 7-hydroxyl group by the 7-*O*-acetyltransferase apparently is not due to a physical process as determined earlier with isolated sialic acids (*29*), but seems to be the result of the activity of a specific enzyme that we would like to tentatively call "sialate:*O*-acetyl ester migrase." Furthermore, the fact that this migration is rapid (migration rate, 3.75%/min),

TABLE III. **Effects of various amino acid-modifying reagents (final concentration, 2 mM) on the acid-insoluble incorporation of radioactivity by Golgi-enriched fractions from bovine submandibular glands.**

Reagent	Modified amino acids	Enzyme activity (% of control)
p-Chloromercuribenzoate	Cys (thiols)	17
Zn^{2+}		53
Cu^{2+}		17
Dithiothreitol	Cys (disulfide)	112
Mercaptoethanol		109
Methylamine	Internal thiol esters	87
Diethylpyrocarbonate	His, Tyr, Lys	7
Acetic acid anhydride	Lys	119
Succinic acid anhydride		104
N-Bromosuccinimide	**Trp**, His, Tyr	72
N-Acetylimidazole	Tyr	55

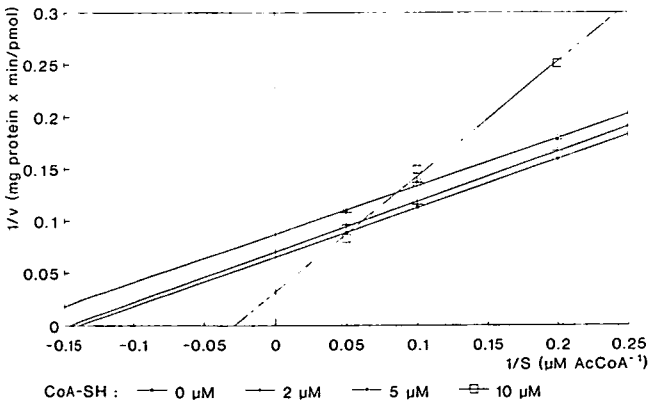

Fig. 8. **Kinetics of the inhibition of acid-insoluble incorporation of radioactivity into microsomes by coenzyme A.** Golgi-enriched fractions (0.5-1 mg protein) were incubated with [^{3}H]AcCoA (0.25 μCi) at varying final concentrations with or without CoA as indicated under "MATERIALS AND METHODS." The quantities of transferred radioactive acetyl groups (pmol) calculated on the basis of the acid-insoluble radioactivity are shown as Michaelis-Menten plots. The K_i value (11.2 μM) was obtained by determination of the slope of the plot, $V'_m - V_m/V'_m$ *versus* I (CoA).

compared to the physical process under similar conditions ($t_{1/2}$ = 600 min at pH 7 and 37°C, corresponding to 0.08%/min) (*29*), is in favour of enzyme-catalyzed migration. The existence of such a mutase has already been discussed by Higa *et al.* (*24*).

Influence of Coenzyme A—Although coenzyme A is a competitive inhibitor of AcCoA in most acetylation reactions, we observed that CoA induced linear, uncompetitive inhibition of the acid-insoluble incorporation of radioactivity into Golgi-enriched fractions with a K_i of 11.2 μM (Fig. 8). This may indicate that CoA does not bind to the free enzyme but only to the preformed Michaelis-Menten complex, as follows:

$$\begin{array}{ccc} & \text{E-I-S} \rightarrow & \text{E + I + P} \\ & \text{I} \leftrightarrow \updownarrow & \\ \text{E + S} \longleftrightarrow & \text{E-S} \rightarrow & \text{E + P} \end{array}$$

where E is the enzyme system (*O*-acetyltransferase and possibly an AcCoA translocator); I, CoA; S, AcCoA; E-S, an acetylated intermediate; and P, *O*-acetylated sialic acids.

This could mean that the *O*-acetylation of sialic acids in Golgi vesicles from bovine submandibular glands occurs at least in two steps, namely, uptake of AcCoA from the incubation medium, followed by the enzymatic transfer of acetyl groups to the glycosidically bound sialic acids in the lumen of Golgi vesicles. This uncompetitive inhibition of the *O*-acetylation of sialic acids by CoA may be explained by the fact that with our enzymatic method the direct effect of CoA on the first step cannot be measured, that on the second reaction being measurable, namely, the transfer of acetyl groups from a hypothetical, acetylated transporter to sialic acids. These considerations are supported by the observation that the acid-soluble incorporation of radioactivity, which also depends on the availability of AcCoA, was independent of the *O*-acetylation reaction of sialic acids.

These findings support the assumption that sialic acids are also *O*-acetylated in submandibular glands through a complex mechanism according to the hypothesis advanced by Higa and co-authors (*24*) that the transmembrane *O*-acetylation reaction occurring in Golgi vesicles from rat liver could be the result of cooperation between an AcCoA transporter, AcCoA hydrolase activity and intraluminal *O*-acetyltransferase activity.

Effect of the Permeabilization of Golgi Vesicles—To further investigate the possibility that the reaction of *O*-acetylation of sialic acids in bovine submandibular glands involves the action of a transmembrane transporter of acetyl groups, we studied the effect of permeabilization of the Golgi vesicles on the rate of 7-*O*-acetylation of sialic acids. As shown in Fig. 9, preincubation of the Golgi membrane fraction with saponin up to a concentration of about 0.01% before the addition of radioactive AcCoA increased the acid-insoluble incorporation of radioactivity by 20%, whereas higher concentrations were inhibitory. It seems that at saponin concentrations lower than 0.01% the Golgi membranes were permeabilized as to AcCoA, resulting in higher *O*-acetylation of sialic acids. It has been shown in various cells (*46-49*) that saponin as a natural steroid interacts with cholesterol in membranes to form 100 nm to 1 μm wide pores, through which the membranes become permeable to ions and other small molecules, but also to various macromolecules such as colloidal gold, catalase and albumin (*50-52*). Thus, our results may be interpreted as showing that AcCoA, a relatively small molecule with a molecular weight of 809 Da, can directly serve as a cosubstrate for the *O*-acetyltransferase, without a transporter, if it has direct access to the lumen of Golgi vesicles. This would be in slight contrast to the model proposed by Diaz *et al.* (*23*) for rat liver. In the latter case, permeabilization of the Golgi vesicles did not have such an effect.

It has to be considered, however, that the results presented, which were reproducible with both total microsomes and Golgi fractions, are due to an artefact. It has been reported (*53*) that the saponin concentration necessary to obtain such pores in membranes is about 0.01% for cholesterol-rich cell membranes, and higher (up to 0.05%) for ER, lysosomes and Golgi membranes. Since the *O*-acetyl transfer was found to be enhanced at saponin concentrations much lower than the minimum concentration required for the permeation of Golgi vesicles (0.05%), this phenomenon may be due to an unspecific effect of saponin on our enzyme test system.

The observation that the non-ionic detergent, Triton X-100, did not significantly influence the acid-insoluble incorporation of radioactivity into Golgi vesicles until its concentration approached the critical micellar concentration (about 0.015%), at which it inhibited the *O*-acetylation (Fig. 9), also indicates an unspecific influence on the AcCoA transporter, the *O*-acetyltransferase itself or the architecture of the membrane structures involved.

Taken together, these results do not contradict the hypothesis proposed for rat liver (*24*) that an AcCoA or acetyl transporter is also involved in the *O*-acetylation of sialic acids in bovine submandibular gland. The strongest evidence for the existence of such a complex system in bovine submandibular gland may be the observation that a pool of acid-soluble, free acetate exists (Figs. 1 and 2). An unequivocal answer to this question is expected on the isolation of the *O*-acetyltransferase and the transporter

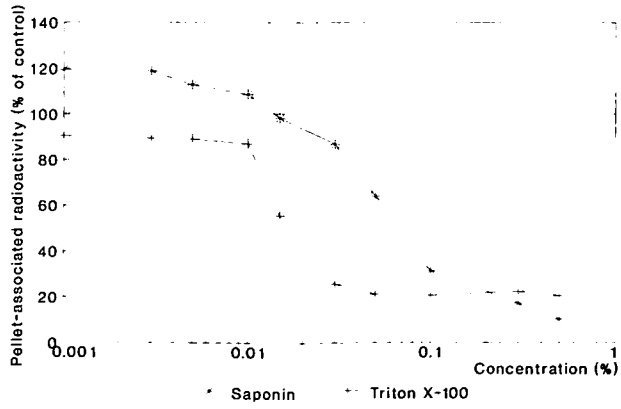

Fig. 9. **Effect of the permeabilization of Golgi vesicles on sialic acid *O*-acetylation.** The Golgi membranes were preincubated for 10 min in 50 mM PK buffer with saponin or Triton X-100 at variable concentrations, and then incubated at 22°C for 20 min with [1-^{14}C]-Ac-CoA under the conditions given under "MATERIALS AND METHODS." The 100% value corresponds to the pellet-associated radioactivity without saponin or Triton X-100.

proteins, and corresponding molecular biological studies. The cloning of a putative AcCoA transporter (*54, 55*) may encourage efforts in this direction.

Effects of Agents Modifying Amino Acids—According to Bame and Rome (*56*) the enzymatic transfer of an acetyl group by acetyltransferases from AcCoA implies the involvement of a thiol group (cysteine), a hydroxyl group (serine, tyrosine), an amino group (lysine), or a nitrogen-containing ring structure (tryptophan, histidine). In order to determine which of these residues is involved in the *O*-acetylation of sialic acid residues in bovine submandibular glands and to confirm the hypothesis according to which the *O*-acetylation investigated with our system could involve a transmembrane enzymatic system, as was described for the *N*-acetylation of glucosamine in lysosomes from rat liver (*56*), Golgi-enriched fractions were treated with different amino acid-modifying reagents.

As shown in Table III, the *O*-acetylation of sialic acids in Golgi fractions was strongly inhibited by *p*-CMB and other heavy metal ions, suggesting that the thiol group from at least one cysteine is involved in the active site of the enzyme. In addition, since oxido-reduction agents (such as dithiothreitol or β-mercaptoethanol) and methylamine did not significantly modify the acid-insoluble incorporation of radioactivity indicates that other sulfur groups, from disulfide bridges or internal thiol esters, are not involved in the enzymatic reaction. As expected, according to the model proposed (*56*), DEPC inhibited the activity strongly (93%), thus indicating the involvement of histidine, tyrosine, or lysine residues. The fact that acetic and succinic acid anhydrides did not influence the acetyl transfer suggests that lysine residues are not located in the reactive site. On the other hand, as shown in Table III, *N*-bromosuccinimide preferentially binding to tryptophan and then to histidine or tyrosine residues resulted in a decrease in the *O*-acetylation rate of only 28%, whereas *N*-acetylimidazole, that only reacts with tyrosine residues, was a stronger inhibitor (about 45% reduction). This seems to indicate that in our system, unlike the system proposed for rat liver (*24*), but in correlation with the *N*-acetylation of glucosamine in the lysosomes of rat liver (*56*), tyrosine residues are more strongly implicated in the catalysis of the bovine sialate-7-*O*-acetyltransferase system than histidine residues. It has, however, to be considered that with the membraneous, heterogeneous system only the translocator and the *O*-acetyltransferase, respectively, or both proteins are modified by these inhibitors.

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