Glycoconjugate J (1988) 5: 257-270

Subcellular Site of the Biosynthesis of *O*-Acetylated Sialic Acids in Bovine Submandibular Gland

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Received November 17, 1987/July 14, 1988.

Key words: O-acetylated sialic acids, O-acetyltransferase. bovine submandibular gland subcellular site, tissue fractionation

Bovine submandibular glands were homogenized and fractionated under conditions which yielded subcellular fragments from mainly one cell type, the mucous acinar cell, as judged by morphological analysis of the glands before and after homogenization. The major *N*-acetylneuraminate-9(7)-*O*-acetyltransferase activity was detected in the cytosolic fraction, a result supported by the high specific radioactivity of free sialic acids isolated after [^{14}C]acetate-labelling experiments. Separation of membranes on a Ficoll density gradient gave six fractions which were analyzed biochemically and morphologically. The particulate activities of acetyltransferase and sialyltransferase were found in fractions containing smooth and mitochondrial membranes. Membrane *O*-acetyl sialic acids were present at the highest levels in these fractions and also had the highest specific radioactivity after [^{14}C]acetate-labelling experiments. Significant amounts of the *O*-acetyltransferase activity also occur in the cytosol and are consistent with a model of *O*-acetyl sialic acid biosynthesis involving both cytosolic and smooth membrane sites of *O*-acetylation.

The occurrence and identification of *O*-acetylated *N*-acylneuraminic acids has been demonstrated in many animal tissues and some microorganisms [1-6]. The ester residues most frequently occur in the glycerol side chains of sialic acids, mainly at C-9, but in some animals, e.g. in the horse, they have also been found in the pyranose ring, at C-4. The animal with the greatest variety of *O*-acetylated sialic acids known is the cow,

Enzymes: Acetyl-CoA:*N*-acetylneuraminate-9(or 7)-*O*-acetyltransferase (EC 2.3.1.45); sialidase or acylneuraminate hydrolase (EC 3.2.1.18); sialyltransferase (EC 2.4.99.3); acid phosphatase (EC 3.1.3.2); alkaline phosphatase (EC 3.1.3.1); NADP-dependent isocitrate dehydrogenase (EC 1.1.1.42); 5'-nucleotidase (EC 3.1.3.5); Na^+; K^+-dependent adenosine triphosphatase (EC 3.6.1.3); β-galactosidase (EC 3.2.1.23).

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in the submandibulary gland glycoprotein of which there occurs a relatively high concentration of *N*-acetyl- and *N*-glycolylneuraminic acid derivatives with one to three *O*-acetyl residues distributed between C-7 and C-9.

Interest in *O*-acetylated sialic acids is increasing, since they have been recognized to play a role in a variety of biological and pathobiochemical processes [7, 8]. For example, they have been identified as essential components of tumor- and differentiation-specific antigens and as the receptor of influence C virus.

Although some evidence for the biosynthesis of *O*-acetylated sialic acids in several cellular systems has been presented [9-15], our knowledge about the subcellular site and other properties of the *O*-acetylating enzymes is still incomplete. Previous work on bovine submandibular gland has suggested that a part of the enzyme activity for *O*-acetylation of sialic acid resides in a microsomal membrane fraction [12], and recent results with cultured liver cells support these findings [14]. The present study is intended to obtain further information concerning the quantity and subcellular localization of this activity in bovine submandibular gland.

The fractionation of subcellular membranes in tissues secreting mucus is difficult due to the "sticky" nature of the glycoproteins. In addition, bovine submandibular glands contain two forms of mucus glycoprotein [16] which are synthesized in different cell types [17]. However, this tissue has been chosen for the study of this *O*-acetyltransferase, since a high activity of the enzyme is expected, due to the large amount of saliva produced.

The work presented here describes a procedure yielding membranes derived predominantly from one cell type and their subsequent fractionation on a discontinuous Ficoll density gradient. A characterization of the membranes associated with the *O*-acetylation of *N*-acylneuraminic acids has been carried out by biochemical and electron microscopical methods.

Materials and Methods

Materials

NADP, AMP, ATP, *p*-nitrophenyl phosphate, isocitrate, coenzyme A, dithio-bis-*p*-nitrobenzoic acid, oxaloacetic acid, citrate synthase and phospho-transacetylase were obtained from Boehringer (Mannheim, W. Germany). Sialidase (*Vibrio cholerae*) was from Behringwerke (Marburg, W. Germany). Glutaraldehyde (25%) and *o*-nitrophenyl-*β*-galactopyranoside were products of Serva (Heidelberg, W. Germany). Ficoll was obtained from Pharmacia (Freiburg, W. Germany). OV-17 on Gas Chrom Q, 100-200 mesh, was from Applied Science (Beijerland, The Netherlands); Dowex 50W and Dowex 2-X8 were products of Fluka (Buchs, Switzerland). NCS tissue solubilizer was from Amersham/Searle (Braunschweig, W. Germany). Glass wool filter papers and ultrathimbles were from Schleicher & Schüll (Düren, W. Germany). Sodium[1-^{14}C]acetate (0.56 mCi/mmol) and [^{14}C]acetyl-CoA (3.7 mCi/mmol) were from Amersham Buchler (Braunschweig, W. Germany). *N*-[^{14}C]acetylneuraminic acid and CMP-*N*-[^{14}C]acetylneuraminic acid (both 0.29 mCi/mmol) were prepared by the methods of Schauer and Buscher [18] and Schauer *et al.* [19], respectively. The other chemicals were of analytical grade and were obtained from Merck (Darmstadt, W. Germany).

Morphology of Intact Bovine Submandibular Glands and Tissue Fractions

Bovine submandibular glands were cut into slices (about 5 × 5 × 1 mm) immediately after removal from the animal and immersed in a fixation solution consisting of 1.5% formaldehyde and 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.8, for 24 h at 4°C. After washing with 0.2 M sucrose in the same buffer for another 24 h at 4°C, the specimens were postfixed in 4% aqueous osmium tetroxide, dehydrated in a graded series of ethanol solutions and embedded in araldite. Semi-thin sections (thickness about 0.5 μm) were cut on a Reichert OMU III ultramicrotome and stained with Mallory's azure II-methylene blue [20] for light microscopy. Ultra-thin sections were cut on the same ultramicrotome, stained with uranyl acetate [21] and lead citrate [22] and investigated using a Zeiss EM9S electron microscope. Samples of the gland tissue for the mophological control of the preparation and fractionation procedure were withdrawn after each step, fixed and embedded as described above.

Fractions obtained after the different centrifugation steps were resuspended and aliquots recentrifuged for 60 min at 100 000 × g to form pellets of about 1 mm thickness. These were fixed as described above while still in the centrifugation tubes and transferred after the washing step into snap-cap vessels for post-fixation and the further embedding procedure. Sections were cut at different positions in a plane perpendicular to the surface of the pellet, to allow for the examination of the total depth of the pellet in a single section.

Preparation of a Crude Membrane Fraction of Bovine Submandibular Glands

A technique was applied which was used earlier for the subcellular fractionation of porcine submandibular glands [23]. Bovine submandibular glands removed 10-15 min after death of the animals at a local slaughter house were cooled on ice and maintained at 2-4°C throughout all subsequent preparation steps, unless otherwise specified. Fat and connective tissue were removed and the glands were diced with a razor blade to approximately 0.5-1.0 cm^3. A batch of 200 g wet weight tissue was usually prepared. After the addition of 50 ml of 0.1 M Tris/HCl buffer, pH 7.2, the tissue was homogenized in an ice bath using an Ultraturrax (Jahnke & Kunkel, Stauffen, W. Germany) for 2 min. This crude homogenate was passed through a tissue press (sieve plate with 1 mm diameter perforations) and frozen at -22°C for at least 12 h, but not longer than 72 h, as 100 g fractions. After thawing to 4°C the 100 g fraction was mixed with 300 ml of 3% Ficoll in the above buffer. This mixture was homogenized with an all glass Potter-Elvehjem apparatus having a clearance of 0.425 mm. A total of ten pestle passages through the mixture were made at 420 rpm pestle rotation. The homogenate was centrifuged at 100 000 × g for 60 min. The cytosolic supernatant was used directly for enzyme assays. The pellet was diluted with 300 ml 1.5% Ficoll in the Tris buffer, re-homogenized as before using a glass pestle and subsequently further homogenized (ten passages at 420 rpm) with a teflon pestle with 0.25 mm clearance. The homogenate was centrifuged at 1 200 × g for 15 min and the floating lipid layer sucked off. The upper loose layer of the sediment was carefully suspended with a spatula so as not to disturb the lower firmly packed layer and was removed by decantation. The decanted suspension was centrifuged for 60 min at 110 000 × g and the pellet suspended in 35 ml of 1.5% Ficoll in the Tris buffer using a Potter-Elvehjem homogenizer with a glass pestle (0.125 mm clearance) for ten strokes at 420 rpm. This membrane suspension, which was almost free of nuclear and connective tissue material, was fractionated on Ficoll density gradients.

Membrane Fractionation on Ficoll Density Gradients

The Ficoll concentrations for the discontinuous gradient were determined from continuous gradient fractionation experiments. The discontinuous gradient consisted of five density layers each of 7-8 ml. These were from top to bottom: 9% Ficoll, refractive index (RI), 1.3492; 15%, RI 1.3581; 17%, RI 1.3613; 19%, RI 1.3649 and 22%, RI 1.3700. The membranes were layered on the gradient as 10 ml of the 1.5% Ficoll suspension to give about 300 mg protein/cm^2 gradient surface in the tube and centrifuged in 3 × 60 ml swing-out-head buckets (WKF RPS 25-2 Hitachi centrifuge) for 2 h at 60 500 × *g*.

The fractions from the discontinuous gradient were carefully withdrawn with a pasteur pipette and resuspended in a five-fold volume of the Tris buffer. The suspensions were pelleted by centrifugation at 100 000 × *g* for 60 min and resuspended in 1-2 ml of the Tris buffer to yield protein concentrations between 50 and 200 mg/ml. Each fraction was ultrasonicated for 15-20 sec using a Branson S 125 sonifier at setting 4. These samples were subsequently investigated for enzyme activity and studied by electron microscopy. The sialic acid content was determined by GLC and Ficoll was preferred to sucrose to avoid difficulties in these analyses.

Incorporation of [1-^{14}C]Acetate into Bound and Free Sialic Acids of Submandibular Glands

Bovine submandibular gland slices (approximately 70 g wet weight) prepared as described earlier [10] were incubated for 4 h with [1-^{14}C]acetate using 7.5 μCi/g tissue. The membrane fraction was isolated from the slices as described above after homogenizing with an Ultraturrax for 2 min and fractionated using the discontinuous Ficoll gradient. The nature and specific radioactivity of the sialic acids in each fraction were analyzed [10, 11].

Measurement of Enzyme Activities

The activities of isocitrate dehydrogenase, 5'-nucleotidase, acid and alkaline phosphatase, β-galactosidase and Na^+, K^+-dependent adenosine triphosphatase were measured as described [23].

Acetyl-CoA:acylneuraminate-9(7)-*O*-acetyltransferase was assayed using exogenous (free *N*-acetylneuraminic acid) or endogenous (membrane-bound or cytosol glycoprotein-bound *N*-acetyl- or *N*-glycolylneuraminic acid) substrate as described by Corfield at *al.* [9]. Sialyltransferase activity was measured using exogenous or endogenous substrates. In the endogenous assay, membrane fractions (5 mg protein in 0.1 ml 0.05 M K_2HPO_4 buffer, pH 7.2, containing 10^{-4} M dithioerythritol) were incubated with 4.5 mCi CMP-*N*-[^{14}C]acetylneuraminic acid [24]. Exogenous sialyltransferase activity was measured in the same incubation mixture as above with 5 mg of desialylated bovine submandibular gland mucin. The radioactive mucin product was isolated and quantified as described by Buscher *et al.* [23]. The linearity of reaction velocity was followed by incubation of the membrane mixture applied to the Ficoll gradient for up to 90 min, and the various membrane fractions were incubated for 20-30 min.

Isolation and Analysis of Sialic Acids

Release of bound sialic acids, including radioactive preparations, from homogenate glycoproteins or the membrane fractions by formic acid hydrolysis, purification over Dowex 50W and 2-X8 ion exchange resins, and cellulose column chromatography, analysis by TLC and colorimetry were performed as described [3, 23].

GLC of the sialic acid fractions was performed on the pertrimethylsilyl derivatives with OV-17 on Gas-Chrom Q, 100-200 mesh, using a Hewlett Packard 5750 G research chromatograph. Standard compounds and operating conditions are described elsewhere [25].

Other Chemical Analyses

Nucleic acids were measured by the method of Schneider [26] and protein by the method of Gornall *et al.* [27]. Acetyl-CoA was measured by a citrate synthase assay [28].

Radioactive Counting

Radioactive samples were counted in 10 ml of a PPO/POPOP/toluene scintillant [24] using a Packard Tri-Carb Model 2450 liquid scintillation spectrophotometer. Where necessary, fractions were solubilized with NCS tissue solubilizer.

Results and Discussion

Morphological Analysis

Light and electron microscopy of the normal glands used in these fractionation studies show the typical morphology of this tissue [29, 30]. Light microscopy of intact glands shows predominantly the terminal portions of the gland tubules (Fig. 1a), embedded in loose connective tissue together with intercalated ducts. The lighter mucous acinar cells are easily distinguished from the darker staining seromucous demilunes. At higher magnification and in the electron microscope (Fig. 1a, insert) light and electron lucent granules (0.5-1.5 μm diameter) can be seen. Serous demilunar cells contain granules of a similar size showing a dense core in light (Fig. 1a, c) and electron microscopy (Fig. 1a, insert). Both types of cells are heavily laden with granules leaving only the basal part of the cells for the nucleus and perinuclear endoplastmic reticulum. In contrast to the work with calf [30] and porcine glands [23], Golgi complexes are inconspicuous or absent. As the animals are starved for at least 12 h before slaughter, the glands are in a resting state physiologically. This may account for the low amount of Golgi membranes observed and the large number of secretory vesicles in both mucous and seromucous cell cytoplasm [17].

Morphological analysis of the diced glands revealed early signs of autolytic destruction (Fig. 1b). The mucous acinar cells are most susceptible to osmotic disruption leading to fusion of mucous granules (Fig. 1b, insert) and fragmentation of intracellular membranes. In contrast, the demilunar cells lose their dense cores and appear osmotically swollen (Fig. 1c), although the surrounding membranes are largely intact. Striated ducts

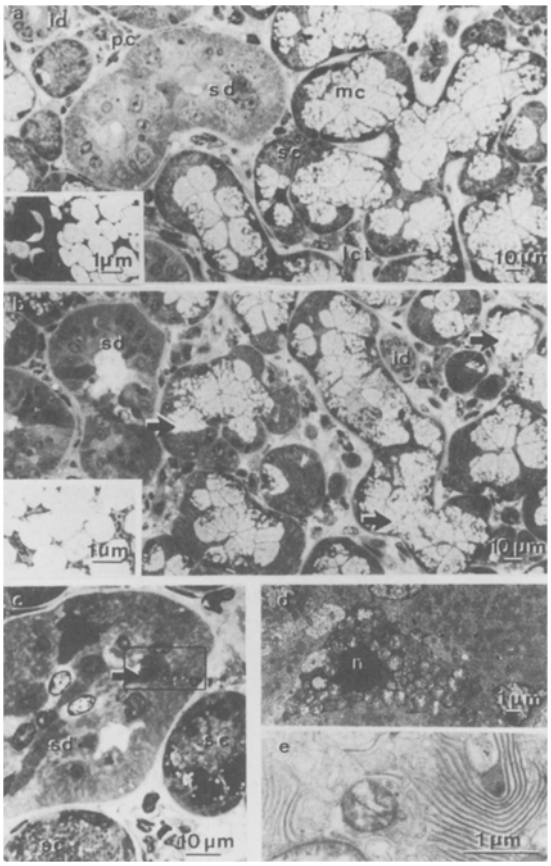

Figure 1. Morphology of intact and diced glands.
(a) Light micrograph of a semi-thin section of the intact bovine submandibular gland. The light mucous acinar cells (mc) are easily distinguished from the darker staining seromucous cells (sc). The terminal portions together with intercalated ducts (id) and striated ducts (sd) are embedded in the loose connective tissue (lct) of the gland stroma, containing fibroblasts and free cells, as e.g. plasma cells (pc). The electron lucent granules of the mucous acinar cells contain flocculent material, while the granules of the seromucous demilunar cells are darker and show a dense core of different size and shape (inset). Magnification: 500-fold, inset: 5 900-fold.
(b) Light micrograph of a semi-thin section of the diced gland, fixed about 6 h after death; this time is necessary, to obtain the gland pieces for the corresponding biochemical procedure. No major alterations are obvious; however, areas showing osmotically damaged intracellular network in several mucous acinar cells (arrows) are visible. Electron microscopy (inset) shows that most of the acinar cell granules have fused and lost their flocculent material. While the intercalated ducts (id) appear largely unchanged, altered cells can be seen in the striated ducts (sd), sometimes staining very darkly. Magnification: 400-fold; inset 8 200-fold.
(c) Another part of the same section as shown in (b) at higher magnification (4 200-fold). One of the dark cells (arrow) with a pycnotic nucleus contains apparently clear vesicles. While the dense cores of the seromucous cells (sc) on the lower side of the photograph are clearly visible, they have largely disappeared in the other seromucous terminal portion.
(d) Electron micrograph of the laelled area shown in (c). The "clear vesicles" inside the cell with the pycnotic nucleus (n) are now recognized as autolytically disrupted mitochondria, situated between stacks of membranes. Magnification: 4 200-fold.
(e) Part of a similar cell as in (d) at higher magnification (21 200-fold). See text for discussion of the figure.

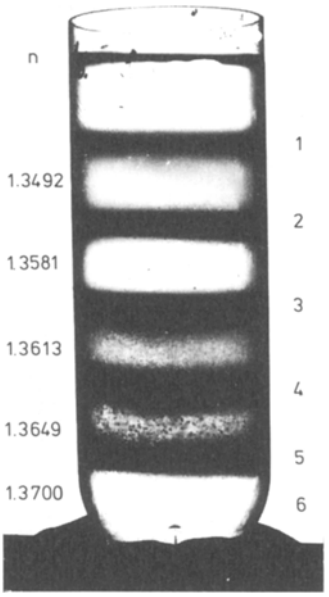

Figure 2. Distribution of subcellular particles from bovine submandibular gland in a discontinuous Ficoll density gradient. The membranes accumulate at the interfaces of the Ficoll concentration layers indicated by their refractive indices, n on the left hand side and the fraction number used in subsequent analysis on the right hand side. The mixed membrane fraction was applied in 1.5% Ficoll to the gradient.

contain several altered cells staining darkly and with clear vesicles (Fig. 1c) which are shown to be disintegrated mitochondria (Fig. 1d). Between the mitochondria, stacks of disc-like saccules resembling Golgi complexes are seen (Fig. 1e). These are likely to be derived from the plasma membranes of the lammellated bases of the striated duct cells [31] during autolysis. Their occurrence exclusively in autolytic cells and at the base of these cells makes it unlikely that they are Golgi membranes and underlines the value of morphological study of intact and diced tissue for the identification of possible artefacts. Such structures found in one of the subcellular fractions (Fig. 3e) may lead to the erroneous assumption that Golgi membranes are present.

The selective disruption of mucous acinar cells observed in the diced tissue (Fig. 1b) was followed further during the preparation using morphometric analysis. Measured values for the different cell types in the homogenate agree well with those calculated on the basis of selective osmotic disruption of mucous acinar cells (Table 1). This result indicates that the membranes obtained by the procedure outlined are predominantly derived from one cell type, the mucous acinar cells.

Tissue Fractionation

The application of classical subcellular fractionation techniques to submandibular gland tissue is much hampered by the presence of viscous mucus glycoprotein [23, 32, 33] and in bovine glands additionally by the dense nature of the tissue. The sequence of homogenization, tissue press and freezing/thawing applied in the present studies

Table 1. Relative volumes of the different cell types in intact and homogenized bovine submandibular gland. Data of intact gland in column 1 are from Veh *et al.* [16] and correspond to the intact organ (Fig. 1a). Data in column 2 correspond to the homogenized gland (Fig. 3a). They were obtained by measuring the relative areas of the individual cell types on micrographs of the homogenized tissue, using a MOP/AM 01 table computer system (Kontron). The relative areas are identical to the relative volumes according to the principle of Delesse [37]. Data in column 3 are calculated theoretically for the homogenized tissue from the data in column 1, assuming the selective fragmentation of 90% of the mucous acinar cells, present in the intact organ. Note the good correlation between columns 2 and 3.

Cell types	Intact gland	Homogenized gland	
		measured values	calculated values
Mucous acinar cells	44%	9%	8%
Seromucous cells and connective tissue	47%	84%	82%
Duct cells	6%	7%	10%

resulted in the dispersion of tissue into membrane fractions derived essentially from the mucous acinar cells. Inclusion of the freezing step proved to be advantageous for the subsequent fractionation procedure. The pellet obtained after recentrifugation of the acinar cell fragments in 1.5% Ficoll consisted of two layers; the lower, firmly-packed layer corresponding to the non-dispersed cellular material and the upper, loose layer to the acinar cell membranes.

This upper layer represents about 3.5% of the original homogenate protein and fractionated best when layered over the gradients at approximately 300 mg protein/cm^2. The results from continuous gradients indicate most membranes occur between RI 1.3581 and 1.3649 with a lighter fraction at RI 1.3492. On the basis of these data a discontinuous gradient was employed between RI 1.3492 and 1.3700 as shown in Fig. 2. Membranes were collected from the interfaces of the layers and reproducibility of the method was established in 21 experiments. Six fractions could be obtained in this way (Fig. 2, Table 2).

Morphological and Biochemical Characterization of the Membrane Fraction

Morphological analysis of the homogenized gland tissue before centrifugation showed about 15% of the total volume to be non-dispersed cellular material (Fig. 3a). This was made up of autolytic demilunar cells surrounded by connective tissue and still containing intracellular granules. Acinar cells were rare and appeared empty. Table 1 shows the relative volumes of these different cells and supports the dispersion of mainly mucous acinar cells, although some duct-cell fragments may also be present. This finely dispersed material constitutes the upper loose layer pelletted during the second centrifugation step (Fig. 3b).

The data for biochemical characterization of the various fractions are given in Table 2. The results indicate that no complete separation of individual membranes was achieved and that the low amount of Golgi complex seen in morphological analysis of the intact glands led to relatively low activities of enzymes associated with this subcellular

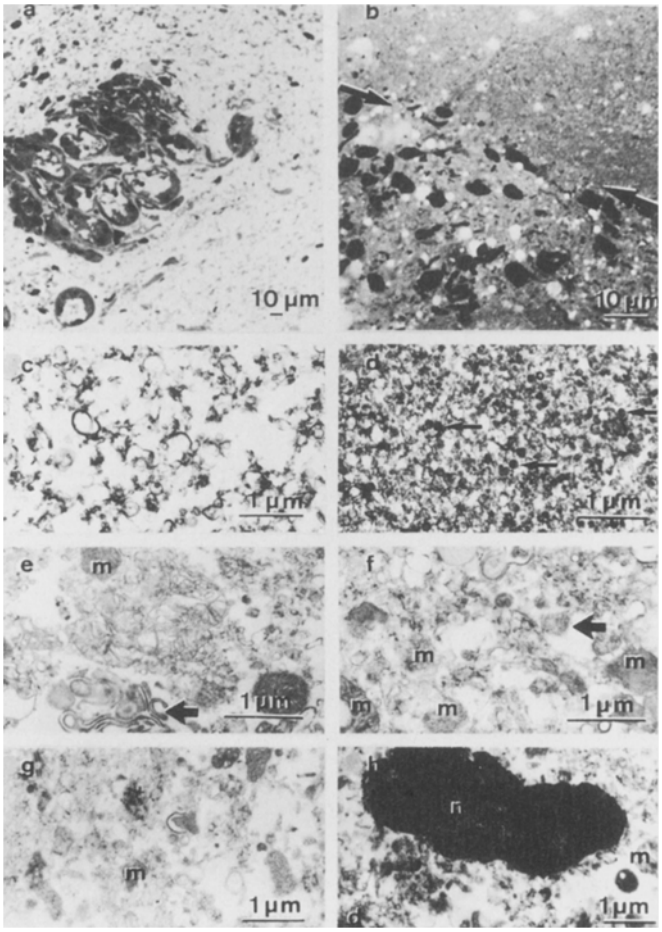

Figure 3. Morphology of the homogenized gland and the subcellular fractions from the discontinuous Ficoll gradient.

(a) Light micrograph of a semi-thin section of the homogenized gland tissue before the first centrifugation step. Magnification: 300-fold.

(b) Light micrograph of a semi-thin section of the pellet after the 1 200 × *g* centrifugation step. A clearly visible boundary (arrows) separates the upper loose layer from the lower layer. Magnification: 1 000-fold.

(c) Electron micrograph of fraction 1 showing membranes and empty vesicles of different size and shape. Magnification: 15 700-fold.

(d) Electron micrograph of fraction 2. The membranes and vesicles are similar to those in fraction 1, however, with a more homogenous appearance. Many of the small vesicles (diameter 0.1-0.15 µm) contain electron dense material (arrows), suggesting a lysosomal origin. Magnification: 16 700-fold.

(e) Electron micrograph of fraction 3. Some mitochondria (m) are found between membrane fragments and empty vesicles. Some dark staining membrane fragments (arrow) resemble "typical Golgi membranes". See text for discussion. Magnification: 19 000-fold.

(f) Electron micrograph of fraction 4. Dispersed between many mitochondria (m) there are membrane fragments, some with adhering mucous material (arrow). Magnification: 18 900-fold.

(g) Electron micrograph of fraction 5. The morphological appearance closely resembles that of fraction 4, except for a lower amount of mitochondria. Magnification: 18 900-fold.

(h) Electron micrograph of fraction 6. Residual cellular material including nuclei (n), mitochondria (m) and desmosomes (d) are seen. Magnification: 12 400-fold.

Table 2. Biochemical characterization of subcellular membrane fractions from discontinuous Ficoll gradient centrifugation. Preparation and other details of the fractions are given in the text, and morphological characterization is presented in Fig. 3. Fractions 1-6 represent in total 20% of the particle mixture before fractionation. Enzyme activities are expressed as mU/mg protein with the exception of exogenous sialyltransferase activity which is expressed in μU/mg protein. The % recovery of each activity is indicated in parentheses relative to the crude homogenate as 100%. Recoveries for fraction 1-6 are relative to the particle mixture before fractionation.

Fraction	Total protein (g)	RNA (pg/mg protein)	Isocitrate dehydrogenase	5'-Nucleotidase	Acid phosphatase	Alkaline phosphatase	β-Galactosidase	Adenosine triphosphatase	*O*-Acetyltransferase		Sialyltransferase	
									endogenous substrate	exogenous substrate	endogenous substrate	exogenous substrate
Crude homogenate	142.8	34	51	10.9	37.3	80.7	2.6	11.4	0.05	0.51	ND[a]	ND
Particle-free supernatant (100 000 × *g*)	13.6(9.5)	10(2.8)	42(7.8)	9.2(8.0)	21.4(5.4)	29.4(3.5)	5.6(20.5)	9.4(7.9)	0.3(57.0)	2.8(52.3)	ND	ND
1200 × *g* Pellet	42.8(30)	80(70)	25(14.7)	9.2(25.3)	59.6(47.9)	120.1(44.6)	2.5(28.8)	15.1(39.7)	0.07(42.0)	0.17(10.0)	ND	ND
Particle mixture before fractionation	5.2(3.6)	12(1.3)	15(1.1)	10.3(3.4)	35.6(3.5)	87.8(4.0)	3.1(4.3)	22.7(7.2)	0.24(17.5)	0.23(1.6)	0.12	7.0
Ficoll gradient fractions 1	0.040	20	5.8	54.3	97.8	86.5	1.6	52.3	0.006	0	0.05	3.3
2	0.111	10	7.0	38.5	161.1	100.9	2.7	11.5	0.16	0.07	0.05	5.0
3	0.275	19	50.9	5.3	34.8	72.3	1.9	72.4	0.46	0.23	0.09	6.6
4	0.120	26	149.7	7.5	31.7	52.9	1.4	98.5	0.62	0.15	0.19	7.7
5	0.070	42	90.8	9.8	26.7	41.6	1.1	64.6	0.51	0.20	0.15	4.9
6	0.300	82	24.6	5.8	45.5	86.7	1.9	14.3	0.35	0.12	0.06	3.3
Recovery (%)	88	302	299	105	137	76	53	186	144	58	65	67

[a]ND = not determined.

fraction. Although the bulk of mucus glycoprotein was removed with the cytosol, the results suggest that some aggregation of membranes due to the presence of residual amounts of these viscous macromolecules occurs. This may be the reason for the poor separation of subcellular membranes from mucous tissues described in the literature [32-34], which is in contrast to the subcellular fractionation of parenchymatous tissues like the liver. The technique described here proved to be the best for the subcellular fractionation of bovine submandibular gland tissue, after much experimental effort including many variations of the methodological conditions had been applied. Similar problems existing in the preparation of subcellular membranes from porcine submandibular glands and the localization of the *N*-acetylneuraminate mono-oxygenase (EC 1.14.99.18) were described earlier [23].

It is shown in Fig. 3 that fraction 1 consisted morphologically of membranes, often fused, which yielded vesicles of different sizes (Fig. 3c). The highest specific activity of 5'-nucleotidase occurred in this fraction, together with significant adenosine triphosphatase activity, indicating plasma membranes. High levels of acid and alkaline phosphatase and β-galactosidase were also present and thus lysosomal membranes were present in this fraction. Fraction 2 contained vesicles (Fig. 3d) similar to those in fraction 1, while marker enzymes suggested a higher lysosomal membrane content. Fraction 3 contained small, empty and irregularly shaped membrane vesicles (Fig. 3e). In addition, mitochondria and the lamellated base plasma membranes derived from striated duct cells were present. This fraction comprised about 30% of the protein recovered from the gradient and contained no maximum for marker enzyme activites (Table 2) but a minimum for 5'-nucleotidase. Fraction 4 contained the highest enzyme marker activities for endoplasmic reticulum membranes and mitochondria. Isocitrate dehydrogenase and adenosine triphosphatase activities derive from mitochondria, while endogenous and exogenous sialyltransferase [34, 35] point to Golgi and smooth membrane fractions. Similar results were found for fraction 5. In these two fractions (4 and 5) mucus-like material could be seen between the vesicles in electron microscopy (Figs. 3f and 3g) and may imply that some of these membranes were from the mucus storage granules and thus originally from Golgi complexes. Mitochondria and striated duct cell membranes could also be seen. The final fraction (6) contained cellular material and connective tissue fibres. Nuclei, mitochondria and desmosomes could also be identified (Fig. 3h), and 65% of the total RNA was present in this fraction (Table 2). The marker enzyme activities reflected the mixture of cells and membranes.

Metabolism of Sialic Acids in Bovine Submandibular Glands

The activities of enzymes metabolizing sialic acids were found in the cytosol (*O*-acetyltransferase) and in the membrane fractions (sialyltransferase and *O*-acetyltransferase). The endogenous and exogenous sialyltransferase activities were concentrated in fractions 3-5 with the highest specific activity in fraction 4. The low enrichment of these enzymes may reflect substrate availability and substrate specificity in the enzyme assay and also the resting state of the glands with low Golgi complex content [33, 35].

O-Acetyltransferase activities tested with endogenous and exogenous substrates occurred both in the particle-free supernatant (cytosol) and in the subcellular membrane fractions (see Table 2). With endogenous substrate, 57% of *O*-acetyltransferase activity present in the homogenate was found in the cytosol, whereas with free *N*-acetylneu-

Table 3. Occurrence and specific radioactivity of glycosidically-bound sialic acids in bovine submandibular gland subfractions. *O*-Acetylated and non-*O*-acetylated derivatives were determined by GLC. Radioactive labelling was carried out by incubation of surviving slices of bovine submandibular glands with radioactive acetate. The *O*-acetylated sialic acids were isolated by preparative TLC. For full description of the procedures see the Materials and Methods section.

Fraction	nMol sialic acid/mg protein	% Recovery	Molar ratio non-*O*-acetylated: *O*-acetylated sialic acids	Specific radioactivity dpm/μg *O*-acetylated sialic acids
Homogenate	9.5	100	1:1.17	13.6
Cytosol	5.6	56	1:1.27	8.5[a]
Particle mixture before fractionation	5.3	2	1:0.85	ND[c]
Ficoll gradient:				
Fraction 1	5.2	3.8[b]	1:0.54	13.3
2	7.1	14.3	1:0.49	25.4
3	7.1	35.4	1:0.69	47.4
4	7.9	17.2	1:0.69	50.5
5	6.1	7.8	1:1.04	86.9
6	11.7	63.7	1:0.75	58.3

[a] Specific radioactivity of free *O*-acetylated sialic acids from the cytosol is 1707 dpm/μg sialic acid.
[b] Recovery of fractions 1-6 is calculated from 20% of particle mixture before loading, and protein concentrations in Table 2.
[c] ND = not determined.

raminic acid 52% of the total enzyme activity was found to be soluble. The recovery of endogenous *O*-acetyltransferase activity greater than 100% in membrane fractions (Table 2) may reflect an underestimation in the homogenate activity or the presence of an esterase activity [7], and is in contrast with the exogenous activity, which is much lower. The activity of the membrane-bound fraction applied to the Ficoll gradient was significantly smaller than the total soluble activity. After fractionation of the membrane pellet on a Ficoll gradient, endogenous and exogenous activities showed a similar distribution to the sialyltransferase with a maximum in fraction 4, and 68% of membrane-bound activity in fractions 3-5.

Data on the sialic acid composition and radioactive acetate labelling of the fractions are shown in Table 3. The highest degree of *O*-acetylation, but combined with the lowest specific radioactivity, was found in cytosolic glycosidically-bound sialic acids, corresponding to the soluble mucus glycoprotein fraction described earlier [10, 11]. This mucus can therefore be considered as mature and stored for secretion. The relatively low degree of *O*-acetylation of sialic acids of the membrane fractions when compared with the cytosol may be due to *O*-acetylation of immature glycoprotein molecules at the site of subcellular membranes or a minor contribution of membrane-bound *O*-acetylation to the total activity. Analysis of individual membrane samples showed that fraction 6 contained most sialic acid (about 45%) probably due to mucus glycoprotein present, while the remaining fractions did not vary greatly from one another. The proportion of

O-acetylated sialic acids in the total membrane-bound sialic acid was highest in fraction 5, one of those containing high *O*-acetyltransferase activity with endogenous substrates. The relatively high radioactive labelling of *O*-acetyl sialic acids in this membrane fraction is in good agreement with these observations.

The finding of both soluble (particle-free supernatant) and membrane-bound *O*-acetyltransferase activities after the fractionation procedure described raises the question whether this activity is present in both forms in the intact gland. No data is at present available to support or refute solubilization of a loosely bound membrane enzyme during homogenization. However, the data are in agreement with earlier studies carried out with surviving slices from bovine submandibular gland [10-12], describing both soluble and membrane-bound enzymes acting on both free and glycosidically bound sialic acids. An important argument for this pathway was the high specific radioactivity of free *O*-acetylated sialic acids, which pointed to a significant level of mucus sialic acid *O*-acetylation before its transer to nascent glycoprotein molecules. This observation is supported in the present studies. Table 3 shows that the specific radioactivity of free *O*-acetylated sialic acid in the cytosol was nearly 20-fold higher than that bound to the membranes of fraction 5, and 200-fold higher than the sialic acids linked to cytosolic glycoprotein. It is assumed that during the process of mucus biosynthesis this sialic acid is transferred to the membranes of fractions 3-5. Further support for a role of a soluble *O*-acetyltransferase acting on free *N*-acetylneuraminic acid (or its CMP-glycoside) comes from the demonstration of the CMP-glycoside of *N*-acetyl-9-*O*-acetylneuraminic acid in bovine submandibular gland [9] and utilization of this glycoside by sialyltransferase from this same tissue [24]. It has been shown here and elsewhere [7] that *O*-acetylation can occur before linkage of sialic acid to CMP, at the level of free *N*-acetylneuraminic acid. However, recent work has provided evidence that this situation observed in bovine submandibular gland may not apply in all cases, as Friend murine erythroleukemia cell *O*-acetylated sialic acids occur exclusively in membrane-bound form, and free *O*-acetylated sialic acids were discussed to arise as a consequence of degradation [36].

The subcellular site of membrane-mediated sialyltransferase leading to mucus glycoprotein is the Golgi complex [34, 35] and the current study suggests that this is one of the possibilities in the bovine submandibular gland. The major site of *O*-acetylation in this tissue appears to be the cytosol, although membrane-bound activity is also demonstrated. Improved fractionation techniques and metabolic labelling methods [10-12, 14, 36] may help to resolve this problem.

Acknowledgements

The authors are indebted to Margret Wember and Ruth Krieger for excellent technical assistance and Dr. H-P. Buscher for valuable discussions. This work was supported by the Deutsche Forschungsgemeinschaft (grants Scha 202/1 and 202/3), the Fonds der Chemischen Industrie, and the Erwin Riesch-Stiftung.

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