Purification and immunofluorescent localization of rat submandibular mucin

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Rat submandibular mucin (RSM) was purified by acid precipitation, then alcohol precipitation of the 30000 g supernatant of gland homogenate, followed by column chromatography on Sephadex G-200. The mucin, which was eluted in the void volume, had an amino acid profile typical of a salivary mucus glycoprotein with high proportions of threonine, serine and proline (48.8% of total amino acids), and low proportions of aromatic and basic amino acids. It consisted of 63% (w/w) carbohydrate, which was shown by g.l.c. analysis to contain *N*-acetylglucosamine, *N*-acetylgalactosamine, galactose, sialic acid and fucose in the proportions 1.0:3.4:2.6:3.1:1.2. After staining of the mucin with periodic acid/Schiff reagent, analytical equilibrium ultracentrifugation in a CsCl density gradient produced a symmetrical peak of buoyant density 1.449 g/ml, without evidence of protein contaminants. Sedimentation velocity centrifugation revealed a major periodate/Schiff-positive component ($s_{20,w}^0$ 5.06) with an associated shoulder of slower sedimenting material, suggesting polydispersity in the size of the mucin. Our findings suggest that the RSM purified in these studies has a molecular weight between 200000 and 1×10^6 . Antibody to RSM was prepared in a rabbit and produced a single precipitin line on immunoelectro-osmophoresis with the mucin. Immunofluorescence studies showed that the antibody localized only to submandibular acinar cells and confirmed that these cells were the source of RSM. The antibody was not directed towards the blood-group-A determinant (terminal *N*-acetylgalactosamine) present in the mucin.

Salivary mucins are viscous secretions of high-molecular-weight glycoproteins whose main function is the lubrication and protection of oral and tracheal epithelium (Herp *et al.*, 1979). The mucins are highly glycosylated, containing 60–80% carbohydrate, which is largely responsible for their physical and chemical properties (Slomiany & Slomiany, 1978). Most of the published work on the purification and characterization of salivary-gland mucins has been done on the glands of animals such as ox, sheep and pig, where large amounts of tissue are available (Tsuiki *et al.*, 1961; Hashimoto *et al.*, 1964; Katzman & Eylar, 1966; Tettamanti & Pigman, 1968; Hill *et al.*, 1977). The salivary mucins of smaller animals, including rodents, are

less well documented and, to our knowledge, the only publication on the isolation of RSM is that of Keryer *et al.* (1973). However, the data provided by these authors are limited, and the carbohydrate and amino acid composition of their RSM is not consistent with those of other reported submandibular mucins (Roukema *et al.*, 1976; Lombart & Winzler, 1972; Oemrawsingh & Roukema, 1974).

The rat submandibular gland is rich in mucous acinar cells and has been extensively used, either in slice or dispersed-cell preparations, as an experimental model for studies on the mechanisms and control of mucus secretion (Bogart & Picarelli, 1978; Quissell & Redman, 1979; Quissell & Barzen, 1980; Fleming *et al.*, 1980; Fleming & Sturgess, 1981). In our laboratory and others, secretion has been assessed by the radioactive labelling of intracellular mucin with the precursor sugar [14 C]-glucosamine, and subsequent measurement of the isotope in the trichloroacetic acid/phosphotungstic acid-insoluble (glycoprotein) fraction of secreted

Abbreviations used: RSM, rat submandibular mucin; IEOP, immunoelectro-osmophoresis; PBS, phosphate-buffered saline (0.1 M-NaCl/0.003 M-NaH₂PO₄, H₂O/0.007 M-Na₂HPO₄, pH 7.2).

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material (Fleming *et al.*, 1980; Quissell & Barzen, 1980). We have now shown, however, by autoradiography (N. Fleming, unpublished work) that [^{14}C]glucosamine is incorporated into the serous secretory granules of cells of the granular convoluted tubule as well as into the mucus granules of acinar cells, so that the radiolabelling technique is not specific for mucin.

The aim of the present study was therefore to purify rat submandibular mucin as the first stage in our eventual goal of developing a mucin-specific assay; and to characterize the mucin more fully than previously reported (Keryer *et al.*, 1973), thereby establishing a background for future studies on secretion and synthesis.

Materials and methods

Purification of submandibular mucin

After removal of the embedded sublingual glands, pooled submandibular glands (18.8 g) from 150 g male Wistar rats were homogenized at 4°C in 20 vol. of 5 mM-disodium EDTA, pH 7.0, in a Sorvall Omnimixer homogenizer at half speed for 30 s. The homogenate was centrifuged at 30 000 g for 30 min, and the clear supernatant was removed and dialysed against four changes in 0.1 M-acetic acid/acetate buffer, pH 4.83, for 48 h (Tettamanti & Pigman, 1968; Herp *et al.*, 1979). Extraneous proteins were precipitated and removed by centrifugation at 2500 g. Ethanol was added dropwise to the supernatant to a concentration of 60% ethanol and the preparation was centrifuged at 30 000 g for 30 min to remove the resulting fine precipitate. The supernatant was adjusted to a final ethanol concentration of 75% (Tettamanti & Pigman, 1968; Herp *et al.*, 1979). A second precipitate was formed, which was retained after 30 000 g centrifugation and dissolved in water. The solution was dialysed against several changes of water for 24 h at 4°C, then freeze-dried, redissolved in 0.1 M-sodium phosphate, pH 7.4, and fractionated by chromatography on a column (65 cm $\times$ 0.9 cm) of Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden). The void-volume (V_0) fraction, which contained carbohydrate, was tentatively assumed to represent purified mucin, and was dialysed against water and freeze-dried. The freeze-dried material was redissolved in PBS to give a concentration of 1.5 mg of protein/ml and stored at -20°C in 50 μ l portions. At each stage of the procedure the concentration of total hexoses [anthrone reaction (Spiro, 1966)] and protein (Lowry *et al.*, 1951) in the preparation were measured and the hexose/protein ratio was determined as a rough index of the purification achieved.

Amino acid and carbohydrate analysis

Samples of purified mucin were hydrolysed in 5.7 M-HCl for 24 h at 110°C under vacuum, then

assayed for amino acids in a Durrum D 500 amino acid analyser. The amino sugars *N*-acetylglucosamine and *N*-acetylgalactosamine were also estimated in the amino acid analyser after partial hydrolysis of samples in 4.0 M-HCl for 7 h at 110°C. Sugars were also determined by g.l.c. of the trifluoroacetate derivatives of *O*-methyl glycosides (Zanetta *et al.*, 1972) in a Varian Aerograph series 2100 gas chromatograph with a 3% OV 210 column. The sugars were quantified by comparison with standards containing 0.1 μ mol of each monosaccharide, and the internal standard inositol (0.02 μ mol) was added to both test and calibration solutions.

Blood-group activity

Purified mucin was examined for blood group activity by a haemagglutination inhibition test as follows. Doubling dilutions of RSM were prepared to give a concentration range of 150 μ g to 73 ng of protein/ml. Samples of 50 μ l of diluted mucin were mixed with 25 μ l of human anti-(blood group A) or anti-(blood group B) antiserum (Pfizer Inc., Diagnostics Division, New York, NY, U.S.A.) in small test tubes and allowed to stand for 30 min. Suspensions of A-group and B-group erythrocytes (5%, v/v, in PBS) were prepared and 50 μ l portions were added to the appropriate test tubes to give the mixtures RSM + anti-A + A cells and RSM + anti-B + B cells. After 1 h the preparations were examined for agglutination.

Analytical density-gradient equilibrium centrifugation

Small samples of mucin (approx. 50 μ g of protein) were stained with periodate/Schiff reagent to produce absorbance readings of about 0.4 at 555 nm. The periodate/Schiff-stained samples were added to a stock solution of 42% (w/v) CsCl in phosphate buffer (33 mM-NaCl/16.7 mM- Na_2HPO_4), pH 6.8, and the density was obtained by refractive-index measurements. Solutions were subjected to analytical density-gradient ultracentrifugation in a Beckman model E ultracentrifuge at 44 000 rev./min for 48 h at 25°C as described by Forstner *et al.* (1979). Buoyant densities were calculated from 555 and 280 nm tracings by using standard formulae (Chervenka, 1970).

Band ultracentrifugation

Purified mucin samples were stained with periodate/Schiff reagent as described above and band ultracentrifugation was carried out in $^2\text{H}_2\text{O}$ -containing self-generating density gradients at pH 5.5 as described by Jabbal *et al.* (1975). Rotor speed was 36 000 rev./min and sedimentation patterns were obtained at 555 nm at 16 min intervals over 4 h. Sedimentation coefficients were calculated from radial displacement of peak apices relative to

time. Values were corrected for solvent density and viscosity (Jabbal *et al.*, 1975).

Immunization of rabbits with submandibular mucin

Four male New Zealand White rabbits were injected subcutaneously at two sites on the back with 40 μ g of purified mucin protein per animal per inoculation. The injection volume was made up to 1 ml by the addition of complete or incomplete Freund's adjuvant and Pertussis vaccine, which was included as a general stimulant of the reticuloendothelial system. The immunization protocol was as follows: day 1, RSM + complete Freund's adjuvant + Pertussis vaccine injected; day 14, day 1 treatment repeated; day 21, blood drawn and serum stored; day 28, RSM + incomplete Freund's adjuvant injected (no Pertussis vaccine); day 40, blood drawn and serum stored. Blood samples from pre-immune and immunized animals were collected from the central ear artery, centrifuged at 1800g and the serum was stored at minus 20°C.

Immunoelectro-osmophoresis

Sera from immunized rabbits were tested for anti-RSM antibody by immunoelectro-osmophoresis, a method that is considerably more sensitive and rapid than Ouchterlony double diffusion (Prince & Burke, 1970; Roitt, 1974). The procedure was as follows. Agarose gel (HEEO agarose; Miles Laboratories, Elkhart, IN, U.S.A.) was prepared at 0.5% (w/v) concentration in 0.05 M-Tris/HCl, pH 8.6, at 100°C, poured on to a glass plate (5 cm $\times$ 7.5 cm) to a depth of about 1 mm and allowed to cool. A series of small wells was cut in two parallel rows 1 cm apart. Samples of purified mucin (12–15 μ l), at the same concentration as inoculated into rabbits (40 μ g/ml), were placed in the cathodal wells and undiluted sera from pre-immune and immunized rabbits in the anodal wells. Electrophoresis was carried out at a constant current of 12 mA (110–120 V) for 75 min in a running buffer of 0.1 M-Tris/HCl, pH 8.6. The gels were washed in 0.1 M-NaCl for 15 min, rinsed in two changes of water and dried at 50°C until clear. They were then stained for 30 min in 0.5% Coomassie Blue to reveal precipitin lines between the wells, destained in several changes of 10% acetic acid and photographed.

Immunofluorescence studies

Unfixed frozen sections of rat submandibular gland were mounted on glass slides and incubated for 45 min at room temperature in sera from pre-immune rabbits, immunized rabbits, normal human or goat. Sera were used at 1:10, 1:20 and 1:40 dilutions in PBS. The sections were rinsed three times with PBS, then incubated for 45 min with goat anti-(rabbit immunoglobulin G)–fluorescein isothio-

cyanate complex (Hyland Laboratories, Costa Mesa, CA, U.S.A.). The preparation was again washed three times with PBS and mounted under a coverslip in 50% glycerol in PBS. The sections were examined in a fluorescence microscope with incident mercury lamp illumination and photographed.

Results

Purification of RSM

In preliminary experiments, rat submandibular gland was homogenized in disodium EDTA and the 30000g supernatant was fractionated on Sephadex G-200 to produce the elution profile shown in Fig. 1(a). The profile consisted of a major glycoprotein peak in the void volume plus a series of included-volume peaks representing extraneous proteins. The

Fig. 1. Sephadex G-200 chromatography of rat submandibular gland homogenate

Rat submandibular glands were homogenized in 5 mM-disodium EDTA/NaOH, pH 7.0. The 30000g supernatant was applied to a Sephadex G-200 column (65 cm $\times$ 0.9 cm) and eluted with 0.1 M-sodium phosphate to produce the profile shown in (a). V_0 indicates the void volume of the column. In separate experiments, the supernatant was subjected to the purification procedure described in the Materials and methods section and the material that precipitated between 60 and 75% ethanol was then fractionated on Sephadex G-200 to produce the profile shown in (b). RSM appeared in the V_0 fraction of (b).

supernatant was then subjected to the purification procedure described in the Materials and methods section and the fraction that precipitated between 60 and 75% ethanol was redissolved and eluted on Sephadex G-200, giving the profile shown in Fig. 1(b). Most of the material was in the V_0 peak, with less than 10% eluted at about 100ml, close to the total volume of the column (Fig. 1b). The hexose/protein ratio in the original 30000 g supernatant was 0.044 and in the V_0 fraction of Fig. 1(b) it had risen to 0.32, representing an 8-fold enrichment of hexose relative to protein. The V_0 fraction of Fig. 1(b) was thus tentatively assumed to represent submandibular mucin. The exclusion limit of Sephadex G-200 is mol.wt. 600000 for peptides and globular proteins, and mol.wt. 200000 for dextrans. As the structure of RSM is unknown, it is proposed that the molecular weight of the mucin is at least greater than the lower of these two limits, i.e. mol.wt. 200000.

Amino acid analysis

The amino acid profile of RSM is shown in Table 1. The mucin contained 37% protein and its composition was comparable with those determined for mucins isolated from mouse submandibular and sublingual, human submandibular and rat sublingual glands (Oemrawsingh & Roukema, 1974; Moschera & Pigman, 1975; Roukema *et al.*, 1976). As is typical for mucus glycoproteins, threonine, serine and proline were present in high concentrations and together comprised 48.8% of the total amino acid content. Tyrosine, phenylalanine, histidine, arginine and methionine were detected in low concentrations

and totalled only 5.5% of all amino acids. RSM differs considerably from canine submaxillary mucin, which has an unusually high content of glycine (22.5%), and a threonine, serine and proline total of 34.5% (Lombart & Winzler, 1972).

Carbohydrate analysis

Blood-group activity. RSM inhibited the agglutination of blood-group-A erythrocytes by anti-A, antiserum. At the concentration of erythrocytes and antisera used, the lowest concentration of mucin causing inhibition was 4.2 µg of protein/ml. Thus the mucin had strong A-blood-group activity, suggesting that it contained at least some *N*-acetylgalactosamine residues linked ($\alpha 1 \rightarrow 3$) to galactose at the non-reducing terminus of oligosaccharide prosthetic groups (Schachter & Roden, 1973). Purified RSM did not inhibit the agglutination of blood-group-B erythrocytes with anti-B antiserum.

G.l.c. sugar analysis. The monosaccharide content of RSM is shown in Table 2. Relative to *N*-acetylglucosamine, we found high amounts of *N*-acetylgalactosamine, which is in keeping with the demonstrated blood-group-A activity. The *N*-acetylgalactosamine/(serine + threonine) ratio was 2.1:1, which is compatible with the possibility that every serine and threonine residue may be linked to an oligosaccharide and that every oligosaccharide may contain an additional A-blood-group *N*-acetylgalactosamine residue as well as an *N*-acetylgalactosamine *O*-glycosidically linked to the peptide core. Galactose and sialic acid were also prominent, as is the case for mouse sublingual mucin (Roukema *et al.*, 1976), but fucose was proportionately higher than in most salivary mucins isolated to date (Moschera & Pigman, 1975; Roukema *et al.*, 1976) except canine submaxillary mucin (Lombart & Winzler, 1972). The sugar profile of our RSM, like the amino acid profile, bore little resemblance to that of RSM reported by Keryer *et al.* (1973).

Calculations based on estimates of protein (Lowry method) and sugar (g.l.c. analysis) content showed that the RSM isolated in our studies consisted of 63% (w/w) carbohydrate, a value within the usual range reported for other salivary mucus

Table 1. *The amino acid composition of rat submandibular mucin*

Amino acid	Content (mol/1000 mol of amino acids)
Asp	87
Thr	246
Ser	109
Glu	80
Pro	133
Gly	60
Ala	75
½Cys	2
Val	27
Met	6
Ile	32
Leu	37
Tyr	10
Phe	14
His	15
Lys	50
Arg	10
Thr + Ser + Pro (mol%)	48.8
Protein (% w/w)	37

Table 2. *The carbohydrate composition of rat submandibular mucin*

Monosaccharide	Molar ratio
GlcNAc	1.0
GalNAc	3.4
Gal	2.6
Sialic acid	3.1
Fuc	1.2
Man	<0.03
Carbohydrate (% w/w)	63

glycoproteins (Lombart & Winzler, 1972; Roukema *et al.*, 1976). Comparable data were unfortunately not provided for the RSM purified by Keryer *et al.* (1973).

Analytical CsCl-density-gradient ultracentrifugation

The glycoprotein profiles of periodate/Schiff-stained RSM after analytical equilibrium ultracentrifugation in a CsCl gradient were monitored by absorption measurements at 280 nm (for peptide) and 555 nm (for carbohydrate) and are shown in Figs. 2(a) and 2(b). In both cases a single symmetrical peak of buoyant density 1.449 g/ml was revealed. It has been shown that the density-gradient ultracentrifugation technique is capable of detecting protein contaminants comprising as little as 0.5% by weight of glycoprotein mixtures (Creeth *et al.*, 1977; Creeth, 1978). Since we saw no evidence of 280 nm-absorbing material of low buoyant density at

the top of the cell, we concluded that the RSM isolated in the present study was free from significant peptide contaminants.

Band ultracentrifugation

In sedimentation-velocity analysis of periodate/Schiff-stained RSM a major component was observed, which had a sedimentation coefficient of 5.06. Although RSM was not necessarily in a 'native' state, having been treated with periodate/Schiff reagent, the sedimentation value is comparable with the reported $s_{20,w}^0$ values of 5.5 for mouse submandibular mucin (Roukema *et al.*, 1976) and of 4.6 (Hashimoto *et al.*, 1964) and 4.8 (Katzman & Eylar, 1966) for porcine submaxillary mucin. The absorbance profile also revealed the presence of a more slowly sedimenting periodate/Schiff-positive shoulder closely associated with the major 5.06 S fraction, which did not emerge as a separate peak. Therefore, no sedimentation coefficient could be determined for it.

Immuno-electro-osmophoresis

Immuno-electro-osmophoresis was carried out with the purified mucin (antigen) and the sera of immunized rabbits (antibody) or preimmune rabbits. A sharp single precipitin line was observed with the serum of rabbit 2 sampled on day 40 of the immunization schedule (Fig. 3). All other sera tested failed to produce precipitin lines with RSM antigen. The poorly-defined lines observed close to the antiserum wells are artefacts produced by the

Fig. 2. Analytical CsCl-density-gradient sedimentation equilibrium ultracentrifugation of RSM

The mucin was stained with periodate/Schiff reagent, added to 42% (w/v) CsCl in phosphate buffer, pH 6.8, and centrifuged in a Beckman model E ultracentrifuge (44 000 rev./min for 48 h at 25°C). Absorption profiles were monitored at 280 nm (a) and 555 nm (b). In each case a symmetrical peak, buoyant density 1.449 g/ml, was observed.

Fig. 3. Immuno-electro-osmophoresis of RSM and anti-RSM antiserum

A 0.5% agarose gel was prepared in 0.05 M-Tris/HCl, pH 8.6. RSM was placed in the cathodal wells and the sera of preimmune or immunized rabbits, sampled on days 21 or 40 of the immunization procedure, in the anodal well. Immuno-electro-osmophoresis was carried out at a current of 12 mA (110–120 V) for 75 min and the gel was stained with 0.5% Coomassie Blue. A sharp precipitin line is observed only with the day 40 serum of rabbit 2. The poorly defined lines near the antiserum wells are artefacts common to the technique.

immunolectric-osmophoresis technique, and have been described by Prince & Burke (1970).

To test the possibility that rabbit 2 inoculated with RSM had produced antibody against the blood-group-A terminal *N*-acetylgalactosamine of the

mucin oligosaccharides, anti-RSM antiserum was mixed with a 2.5% suspension in PBS of blood-group-A erythrocytes. No agglutination was observed, and it was concluded that blood-group-A was not an antigenic determinant for the RSM antibody.

Fig. 4. *Immunofluorescence of RSM*

(a) Fresh frozen sections of rat submandibular gland were incubated with rabbit anti-RSM antiserum, then exposed to fluorescent labelled second antibody [goat anti-(rabbit immunoglobulin G)-fluorescein isothiocyanate]. Fluorescence is observed only in the acinar cells (A) confirming this cell type as the source of RSM. The ductal cells (D) of the gland are negative. Magnification $\times 360$. (b) Section of rat submandibular gland incubated with preimmune serum followed by fluorescent labelled second antibody. No fluorescence is observed in either acinar (A) or ductal (D) cells. Magnification $\times 360$.

Immunofluorescence studies

When rat submandibular-gland sections were incubated with anti-RSM antiserum, then exposed to fluorescent-labelled second antibody, it was observed that fluorescence occurred only in the acinar cells (Fig. 4a). No fluorescence was detected in the various epithelial cell types lining the duct system of the gland, i.e. intercalated duct, granular convoluted tubule, striated duct and excretory duct. Thus the anti-RSM antibody was specific for acinar cell contents. As acinar cells are the mucin-secreting component of rat submandibular gland, it was therefore confirmed that the fraction of the gland that had been isolated as purified RSM and used to immunize rabbits had indeed originated in mucous cells.

Sera from a pre-immune rabbit (Fig. 4b) or from human or goat (results not shown) did not produce fluorescence.

Discussion

In the present study we describe the isolation and characterization of rat submandibular mucin. Several criteria suggest that the RSM is a highly purified preparation. The negligible amount of mannose confirmed that the mucin was not significantly contaminated with membrane or plasma glycoproteins, and CsCl-density-gradient equilibrium ultracentrifugation of periodate/Schiff-stained mucin showed only a single component of buoyant density 1.499 g/ml, with no peptide contaminants. Immunofluorescence studies demonstrated that anti-mucin antibody localized only to the mucous acinar cells of the gland; connective tissue was not stained. Immunoelectro-osmophoresis revealed a single sharp antigen-antibody precipitin line. The fact that preimmune serum was negative in both the immunofluorescence and immunoelectro-osmophoresis studies again demonstrates that RSM antigen was free from serum contaminants.

Sedimentation velocity centrifugation of periodate/Schiff-stained RSM produced a profile that suggested polydispersity in size of the mucin. A major component ($s_{20,w}^0$ 5.06) was observed, with lower molecular-weight material appearing as a shoulder. In buoyant-density studies, however, a more unimodal density distribution was obtained, indicating that this technique does not necessarily detect size differences. For both sedimentation and density studies RSM was stained with periodate/Schiff reagent before analysis. It was shown in a previous study (Jabbal *et al.*, 1975) that exposure of rat intestinal mucin to periodate/Schiff reagent under very mild conditions caused modifications of terminal sialic acid and fucose residues, but did not produce gross physical or chemical changes in the mucin as assessed by several criteria, including

Sephacrose 4B column chromatography, protein analysis, electrophoretic mobility in polyacrylamide gel, immunoprecipitation by anti-mucin antibody and measurement of sedimentation coefficient. In the present study, possible periodate/Schiff-induced modifications of RSM were not monitored. Thus some caution should be exercised in assuming that the results faithfully reflect the exact properties of intact RSM. It has been shown by Scott *et al.* (1972) and Scott & Tigwell (1973) that certain proteoglycans and glycosaminoglycans lose viscosity after treatment with 0.1M-periodate, an effect that has been attributed to the rupture of glycosidic links by the generation of free hydroxy radicals from periodate. In principle, any polysaccharide polymers might be susceptible to the same glycosidic bond breakage although no study of mucin oligosaccharide chains has been performed to confirm it. In general the sedimentation and density profiles of periodate/Schiff-stained RSM are compatible with the high molecular weight and polydispersity usually reported for intact salivary mucins. For example, ovine and bovine submaxillary mucins were observed by Tettamanti & Pigman (1968) to have a major and a minor component; and salivary mucins in general have considerable polydispersity of size (Payza *et al.*, 1970). Hashimoto *et al.* (1964) reported that porcine submaxillary mucin had a sedimentation coefficient of 4.6, representing a mol.wt. of 830 000. Assuming that the value of 5.06 after staining reflects fairly accurately the $s_{20,w}^0$ of the major component of unstained or intact RSM, we expect that RSM may be in the same size range. Since RSM was excluded by Sephadex G-200 but included by Sepharose 4B (results not shown), the average molecular weight probably lies between 200 000 and 1×10^6 .

It is assumed (but not proven) that much of the polydispersity in mucins can be attributed to differences in length, composition or charge of sugars in the oligosaccharide side chains rather than to variations in the amino acids of the protein core. The assumption is supported by the fact that the sugar content of salivary mucins from different glands and species shows much more variation than do the amino acid profiles (Lombart & Winzler, 1972; Oemrawsingh & Roukema, 1974; Moschera & Pigman, 1975; Roukema *et al.*, 1976). The high *N*-acetylgalactosamine/(serine + threonine) ratio (2.2:1) of RSM suggests that there may be, on average, two *N*-acetylgalactosamine residues per oligosaccharide chain. However, the sugar composition of the mucin does not permit the construction of a single average oligosaccharide chain type that could account for all the monosaccharide ratios observed. Moreover, our preparation has high concentrations of both fucose and sialic acid, which generally have an inverse relationship in oligo-

saccharide chains (Herp *et al.*, 1979). This probably means that RSM has several types of sugar chains, some long and containing more fucose, some short and containing more sialic acid. Oligosaccharide heterogeneity in RSM may be similar to that of rat sublingual mucin, which is reported to contain from 9 to 15 monosaccharide residues per chain (Slomiany & Slomiany, 1978).

The high content of sialic acid in our RSM preparation is not compatible with the histochemical observation by Spicer & Warren (1959) that the gland is low in sialic acid. It is possible that histochemical techniques used by these investigators did not accurately reflect the sialic acid content of the mucin. It has been demonstrated by Culling *et al.* (1979) that sialic acid residues of human intestinal mucin display variations in their histochemical staining characteristics, depending on the degree of *N*- and *O*-acetyl substitution of hydroxy groups, which leads to a loss of vicinal diols in the molecules. On this basis we would predict a high content of di-acetylated neuraminic acid residues in RSM.

When the composition of RSM prepared in these studies was compared with that isolated by Keryer *et al.* (1973), there were several discrepancies. For example, in their carbohydrate profile, fucose was absent, and *N*-acetylgalactosamine and sialic acid were low relative to *N*-acetylglucosamine; whereas in our preparation, fucose is present and *N*-acetylgalactosamine and sialic acid are high relative to *N*-acetylglucosamine. In amino acid composition our RSM has a profile similar to those of other salivary mucins, with a high content of serine, threonine and proline residues (Oemrawsingh & Roukema, 1974; Moschera & Pigman, 1975; Roukema *et al.*, 1976). The amino acid profile of the RSM prepared by Keryer *et al.* (1973) was not typical of a salivary mucin since it had a lower concentration of serine, threonine and proline residues and higher content of aspartic acid, glutamic acid and glycine residues. Few criteria of purity were given by these authors, and Moschera & Pigman (1975) speculated on the possibility of contamination of the RSM prepared by Keryer *et al.* (1973) by glycoproteins from the embedded sublingual glands. In the present study, the sublinguals were removed before purification of RSM, so there is no possibility of such contamination. Finally, differences between the two mucins may be due to variations in purification procedure selecting for different products. The technique of Keryer *et al.* (1973) consisted of fractional precipitation with ethanol, followed by zone electrophoresis on Pevikon at pH 6.0. The latter step has not been tried in our experiments, so its contribution to purification and composition is unknown.

The antibody to RSM did not react with blood-

group-A erythrocytes, despite the high *N*-acetylgalactosamine content of the mucin antigen. This is consistent with the observation that antibodies directed against human submandibular mucin (Oemrawsingh & Roukema, 1976) or human intestinal goblet-cell mucin (Qureshi *et al.*, 1979) do not react with blood-group substances. This is an interesting finding since mucins are often rich in ABH and Lewis determinants (Schachter & Roden, 1973), are quite antigenic and might therefore be expected to elicit antibodies specific for blood-group determinants. Discovering the identity of antigenic determinants in native mucin may provide an important probe of mucin structure and is a goal of future studies.

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