Carbohydrate Research, 40 (1975) 53–67

THE ISOLATION AND CHARACTERIZATION OF RAT SUBLINGUAL MUCUS-GLYCOPROTEIN*

JOHN MOSCHERA AND WARD PIGMAN

New York Medical College, Biochemistry Department, Valhalla, New York 10595 (U. S. A.)

(Received October 29th, 1974; accepted in revised form, December 16th, 1974)

ABSTRACT

A purified glycoprotein, designated RSL-major, was isolated from the rat sublingual gland by means of the procedure of Tettamanti and Pigman. It was found to be homogeneous by analytical ultracentrifugation, to have a mol. wt. of 2.2×10^6, and to contain 81% (w/w) of carbohydrate, which consists mainly of sialic acid, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, and D-galactose in the molar ratio of 1.4:1.4:1.0:1.5; small amounts of fucose and mannose [1.2 and 2.8% (w/w), respectively] were also present. The sialic acid residues were resistant to the action of *V. cholerae* neuraminidase. This resistance was completely abolished by removal of the *O*-acetyl groups contained in the sialic acid. The sialic acid in RSL-major appeared to be a mixture of *N*-acetyl-4-*O*-acetyl- and *N*-acetyl-4,7(8)-di-*O*-acetylneuraminic acids. The carbohydrate to protein attachment of RSL-major was shown, by alkaline β-elimination reaction, to consist of an *O*-glycosyl linkage between 2-acetamido-2-deoxy-D-galactosyl residues in the oligosaccharide chains and seryl and threonyl residues in the protein core. The average oligosaccharide, contained in RSL-major, was postulated to be a heptasaccharide. A second material, designated RSL-minor, and also isolated from the rat sublingual gland, was obtained as a mixture of glycoprotein(s) and hydroxylapatite gel, and was not purified further.

INTRODUCTION

The epithelial mucins are the glycoproteins responsible for the viscous nature of the mucus produced by the oral, respiratory, gastrointestinal, and reproductive tracts of higher animals. The best characterized of these materials are the mucus glycoproteins derived from bovine, ovine, and porcine submaxillary glands, human and porcine gastric mucosa, bovine cervix, and human saliva. Recent reviews of the epithelial mucus glycoproteins have been published[1–4].

Studies of the biosynthesis of mucus glycoproteins and of their physiological function have been handicapped because sources of the most well characterized materials are either not readily available, or are derived from animals that cannot be conveniently handled in the laboratory. For this reason, we decided to investigate the

* Dedicated to Professor Michael Heidelberger in honor of his 87th birthday.

salivary mucus glycoproteins produced in an animal that would be both easily obtainable and of a size suitable for handling. Initially, we selected the rat submaxillary gland for this purpose, but soon found that the mucus glycoproteins derived from this source were mainly the products of the small sublingual gland, which lay embedded in the submaxillary tissue. This observation is in agreement with previously obtained histological data which indicates, that while the rat sublingual gland is rich in sialic acid-containing mucus glycoproteins[5–7], the rat submaxillary gland contains very little sialic acid[5].

EXPERIMENTAL

Isolation of material. — Rat submaxillary glands were obtained, as a mixture of sublingual and submaxillary, from Pel Freeze Biologicals, Inc., Rogers, Arkansas 72756. The glands were removed from the adult animals (Wistar, Sprague-Dawley, and descendants), quickly frozen in pairs, and shipped to us in dry ice. They were kept at −10° until ready for use, at which time they were thawed at 4° in a cold room.

The rat sublingual glycoprotein was isolated essentially according to the procedure described by Tettamanti and Pigman[8] for the preparation of bovine and ovine submaxillary glycoproteins. For our purposes, the procedure was modified in the following way: (a) The rat sublingual glands (50 glands, 2.5 g wet weight) were homogenized in two 50-ml portions of 0.01M sodium chloride solution by means of a "Duall" tissue grinder (Kontes, Vineland, New Jersey, 08360). (b) The combined saline supernatants were acidified to pH 4.85 rather than to pH 4.7, as originally used. (c) The sialic acid-containing material, dissolved in 50% (w/v) aqueous calcium chloride, was precipitated at an ethanol concentration-range of 70–75% (v/v).

The major glycoprotein (RSL-major), isolated by this procedure, was obtained in an average yield of 2.2% of the wet weight of the tissue used. A second material (RSL-minor), which corresponded to the minor amount of mucin of Tettamanti and Pigman[8] in adsorption to hydroxyapatite, was also obtained, but in a yield of only 0.6%.

Analytical methods. — The protein content of samples was measured by the method of Lowry *et al.*[9] with bovine serum albumin as a standard, and also by calculation of the weight contribution of amino acids, as determined by amino acid analysis. Amino acids were analyzed on a model 120B amino acid analyzer (Beckman Instruments Inc., Fullerton, Calif. 92634) under conditions found to be suitable for mucus glycoproteins[10].

Total hexosamine content was determined by the Elson–Morgan reaction as modified by Blix[11], with values being reported relative to 2-amino-2-deoxy-D-glucose. Samples were hydrolyzed in 6M hydrochloric acid for 4 h at 106°. The hydrolyzates were evaporated to dryness in a vacuum desiccator over sodium hydroxide, and the residues dissolved in a suitable volume of water for analysis. The differential determination of 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose was achieved on the amino acid analyzer by use of a 15-cm column of Beckman 50A resin, maintained at 51°. The column was eluted with a sodium citrate buffer (pH 5.25,

0.35M in sodium ion) at a flow rate of 40 ml/h. Under these conditions, the elution times for 2-amino-2-deoxy-D-glucose and 2-amino-2-deoxy-D-galactose were 45 and 51 min, respectively.

Sialic acid was routinely determined by the modified resorcinol method of Svennerholm[12,13] with *N*-acetylneuraminic acid as a standard. The results were corrected for *O*-acetyl content, as measured in representative samples by the alkaline hydroxylamine procedure of Hestrin[14], for which α-D-glucose pentaacetate served as a reference material. Free sialic acid was measured after acid hydrolysis by the resorcinol method[13] and by the thiobarbituric acid method of Warren[15]. The samples were dissolved in water (1–3 mg glycoprotein per ml), adjusted to pH 2.0 with 0.5M sulfuric acid, and heated for 90 min at 85°. After being cooled, the hydrolyzates were applied to a column (0.5 × 8 cm) of Bio-Rad AG1-X8 ion-exchange resin (HCO_3^-, 50–100 mesh) and eluted according to the method of Svennerholm[16], as described by Spiro[17]. The eluates were evaporated to dryness in a rotary evaporator at 30°, and the respective residues dissolved in a suitable quantity of water for analysis. The *N*-glycolyl content was determined by the method of Klenk and Uhlenbruck[18].

T.l.c. of the free sialic acid fraction of RSL-major, obtained as just described, was achieved on microcrystalline cellulose plates ("Q-2", Quantum Industries, Fairfield, N. J. 07006) with a developing solvent[19] of 1-butanol–1-propanol–0.1M hydrochloric acid (1:2:1, v/v). Spots were detected with the Ehrlich spray reagent[19]. Under these conditions, *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid had R_F values of 0.46 and 0.37, respectively.

Neutral sugars were identified by means of paper chromatography through the courtesy of Mr. Albert Wu, of our department. Samples of glycoprotein (3–4 mg) were hydrolyzed in screw-capped tubes with 2M hydrochloric acid for 2 h at 100°, and then neutralized with dry Bio-Rad AG1-X8 (HCO_3^-, 100–200 mesh) ion-exchange resin. After filtration, the solution was passed through a column (0.5 cm × 10 cm) of Bio-Rad AG50-X8 (H^+, 100–200 mesh) ion-exchange resin and then evaporated to dryness. The residue was dissolved in a few drops of water and applied to a sheet of Whatman No. 1 chromatography paper. The chromatogram was developed for 72 h with 1-butanol–acetic acid–water (4:1:5, v/v). Spots were detected by staining with alkaline silver nitrate solution[20]. L-Fucose, D-galactose, D-mannose, and D-xylose were used as reference compounds.

D-Galactose and L-fucose were quantitatively determined with the respective methods of Dische[21,22]. Several samples were also analyzed for neutral sugars by g.l.c.[23], through the cooperation of Dr. W. Niedermeier (University of Alabama Medical Center).

The sulfate content was determined by the Antonopoulos procedure[24] and the uronic acid content by the carbazole method[25].

Physical methods. — The intrinsic viscosity at zero concentration was determined at 30° in a 0.5-ml Cannon–Fenske viscometer No. 100 in M sodium chloride solution.

Sedimentation equilibrium experiments were performed on a Beckman Spinco "Model E" analytical ultracentrifuge, as described by Payza *et al.*[26]. Samples, dissolved in M sodium chloride, at a concentration of about 0.2 mg of glycoprotein per ml of solution, were centrifuged for 24 h at 20° with a double-sector 12-mm interference centerpiece, at a speed of 2994 r.p.m. A partial specific volume of 0.633 was used for the calculation of the mol. wt.[27,28].

Alkali treatment. — The alkali β-elimination reaction was performed under the conditions found to be optimal for the release of the carbohydrate moiety from the protein core of bovine submaxillary mucus-glycoprotein[29,30]. Samples of glycoprotein (4 mg) were dissolved in alkaline borohydride solution (2.5 ml, 0.3M sodium borohydride in 0.1M sodium hydroxide), and incubated at 45° in screw-capped tubes. After 5 and 10 h of treatment, aliquots (1 ml each) were transferred to 18 × 150 mm screw-capped tubes and were allowed to cool to room temperature. After the addition of 0.08M palladium chloride (purified according to the procedure of Tanaka and Pigman[31]) and a drop of 1-octanol to each tube, the solutions were neutralized with 4M hydrochloric acid (0.8 ml), added dropwise with vigorous stirring. Reduction and amino acid analysis were performed with the method of Downs *et al.*[30].

For the investigation of the oligosaccharide chains of RSL-major, the glycoprotein was also subjected to the β-elimination reaction under the conditions of Carlson[32,33], which have been found to limit the extent of the secondary peeling-reaction. Samples of glycoprotein were treated for 16 h at 45° with an alkaline borohydride reagent made of 0.05M sodium hydroxide and M sodium borohydride.

For the determination of 2-amino-2-deoxyglucitol and 2-amino-2-deoxygalactitol, the alkali-treated samples were neutralized with dilute hydrochloric acid and evaporated to dryness in a rotary evaporator. Boric acid was removed as its volatile methyl ester by several distillations with 10 ml each of absolute methanol, and the residue dissolved in water for analysis. Hexosamines and hexosaminitols were determined with the amino acid analyzer equipped with a 56-cm column of Beckman 50 A resin, maintained[34] at 65°. Elution was carried out at a flow rate of 40 ml/h with a citrate buffer (pH 5.28, 0.35M Na^+) to which had been added boric acid[35] so as to produce a final borate concentration of 0.2M.

Alkaline hydroxylamine treatment. — A sample of glycoprotein (7 mg) dissolved in water (1 ml) was treated at room temperature with alkaline hydroxylamine reagent[14] (2 ml), prepared by mixing equal volumes of 2M hydroxylamine hydrochloride and 3.5M sodium hydroxide. After 2 min, the solution was neutralized with 33% (v/v) hydrochloric acid (1 ml), dialyzed against distilled water at 4° for 2 days with frequent changes, and lyophilized.

Neuraminidase treatment. — The *V. cholerae* neuraminidase was obtained from General Biologicals (Chagrin Falls, Ohio 44022) and was used at a concentration of 3 units per ml. Solutions of rat sublingual glycoprotein or of its alkaline hydroxylamine-treated derivatives in water (1 ml) were added to 0.05M sodium acetate buffer (0.25 ml, pH 5.5), and incubated with 0.1 ml of enzyme for 24 h at 37°. The free sialic acid of the incubation mixtures was determined by an alkaline Ehrlich procedure[36].

RESULTS

Isolation. — The procedure of Tettamanti and Pigman[8], which is the method of choice for the preparation of bovine and ovine submaxillary-mucins, was used for the isolation of rat sublingual mucus-glycoprotein. As with the bovine and ovine materials, a mild acidification of the saline extracts was found to be a necessary prerequisite to the formation of a completely soluble Cetavlon–glycoprotein clot. However, in the case of the rat material, considerable losses of glycoprotein occurred at a pH lower than 4.85.

The principal glycoprotein (RSL-major) accounted for 85% of the non-hydroxyapatite-treated material. It showed one peak in the ultracentrifuge pattern, and gave a linear Yphantis plot for $\ln c$ *vs* $x^2 - m^2$. These results indicate homogeneity with respect to mol. wt. The mol. wt. calculated from this plot, based on a partial specific volume of 0.633, was 2.2×10^6. The limiting intrinsic viscosity in M sodium chloride was 2.21 dl/g. The plot of η_{sp}/c *vs* c was linear over the range of 0.15–0.44 g/100 ml.

Carbohydrate and amino acid components. — The chemical compositions of the major and minor sublingual glycoproteins are given in Table I. The analytical data in Tables I–III are the average of at least 3 analyses of 3 preparations. The principal carbohydrate components of both materials are sialic acid, 2-amino-2-deoxy-D-glucose, 2-amino-2-deoxy-D-galactose, and D-galactose. Protein content, as measured

TABLE I

CHEMICAL COMPOSITION OF MAJOR AND MINOR RAT SUBLINGUAL GLYCOPROTEINS

Component	*Composition*[a]			
	(g/100 g)		*(mmol/100 g)*	
	Major	*Minor*	*Major*	*Minor*
Sialic acid[b]	32.6	21.9	86.9	58.4
GlcNAc[c]	17.1	10.3	84.2	50.7
GalNAc[c]	12.4	5.9	61.1	29.1
D-Galactose	14.9	9.0	92.0	55.6
L-Fucose	1.2	0.5	8.2	3.4
D-Mannose	2.8	1.5	17.3	9.3
Protein[d]	16.1	19.1		
Protein[e]	17.3	16.8		
Uronic acid	0			
Sulfate	0			
Total recovery[f]	98.3	65.9[g]		

[a]Carbohydrate content is reported as g of anhydro sugar per 100 g of dry material. [b]Reported as *N*-acetyl-di-*O*-acetylneuraminic acid. [c]Abbreviations: GlcNAc, 2-acetamido-2-deoxy-D-glucose; GalNAc, 2-acetamido-2-deoxy-D-galactose. [d]By Lowry *et al.*[9] procedure. [e]By amino acid analysis. [f]Sum of carbohydrate content and amino acid analyzer protein content. [g]Not corrected for ash content.

TABLE II

AMINO ACID COMPOSITIONS OF MAJOR AND MINOR RAT SUBLINGUAL GLYCOPROTEINS

	mmol/100 g		*mol/100 mol*	
	RSL-Major	*RSL-Minor*	*RSL-Major*	*RSL-Minor*
Lys	5.3	7.4	3.0	4.4
Arg	2.8	3.2	1.6	1.9
Asp	12.9	14.9	7.2	8.9
Thr[a]	40.1	26.2	22.3	15.6
Ser[a]	32.6	24.9	18.1	14.9
Glu	12.2	19.5	6.8	11.6
Pro	13.5	11.2	7.5	6.7
Gly	15.7	15.3	8.7	9.1
Ala	21.8	17.9	12.1	10.7
Val	10.6	9.3	5.9	5.6
Ile	3.2	3.6	1.8	2.2
Leu	5.2	6.6	2.9	3.9
Cys[b]	1.5	0.8	0.8	0.5
His	0.8	1.5	0.5	0.9
Tyr	0.3	2.3	0.2	1.4
Phe	1.2	3.0	0.7	1.8

[a]The values reported for serine and threonine have been increased by factors of 10% and 2.7%, respectively, to correct for destruction due to acid hydrolysis. These correction factors were determined experimentally for RSL-Major by extrapolation of 22-, 48-, and 72-hour hydrolysis data. [b]Determined as cysteic acid.

TABLE III

RELATIVE MOLAR COMPOSITIONS OF MAJOR AND MINOR RAT SUBLINGUAL GLYCOPROTEINS[a]

Component	*RSL-Major*	*RSL-Minor*
Sialic acid	48.8	34.8
GlcNAc[b]	46.9	30.3
GalNAc[b]	34.0	17.4
D-Galactose	51.2	33.2
L-Fucose	4.6	2.0
D-Mannose	9.6	5.5
Aspartic acid	7.2	8.9
Glutamic acid	6.8	11.6
Serine	18.1	14.9
Threonine	22.3	15.6
Aromatic amino acids	0.9	3.2
Basic amino acids	5.1	7.2
Total carbohydrate	195.1	123.2
GlcNAc/GalNAc[b]	1.4	1.7

[a]Data presented in terms of mol of component per 100 mol of total amino acids. [b]For abbreviations, see Table I.

by the method of Lowry *et al.*[9], was in good agreement with that determined through amino acid analysis.

Paper chromatography of the neutral sugar fraction of hydrolyzed RSL-major indicated that the principal neutral sugar present was D-galactose. Small amounts of D-mannose and L-fucose were also evident. This result was confirmed by quantitative colorimetric analyses of D-galactose and L-fucose, and by quantitative analysis of D-mannose, as its alditol acetate, by g.l.c.

The differential hexosamine determination indicated that, for both glycoproteins, 2-amino-2-deoxy-D-glucose was present in a higher concentration than 2-amino-2-deoxy-D-galactose. The ratios of the two were found to be 1.4:1 for RSL-major and 1.7:1 for RSL-minor.

T.l.c. of the sialic acid component of RSL-major indicated the presence of *N*-acetylneuraminic acid and two unidentified materials (R_F 0.65 and 0.67) which probably correspond to *O*-acetyl derivatives. The presence of *O*-acetyl derivatives of neuraminic acid was established by means of the alkaline hydroxylamine assay, which indicated a ratio of 1.6 mol of *O*-acetyl residue per mol of sialic acid for the intact glycoprotein. A slightly higher molar-ratio was found for RSL-minor. Although no *N*-glycolylneuraminic acid could be demonstrated chromatographically, the sialic acid derived from RSL-major was found to contain approximately 2.4% of this compound by direct analysis[18].

The amino acid composition of RSL-major and -minor is given in Table II. Serine and threonine, which together account for 40% of the protein core of RSL-major and 30% of RSL-minor, are the two most abundant amino acids present. Both glycoproteins contain only small proportions of the basic, aromatic, and sulfur-containing amino acids.

As with the minor glycoproteins isolated from bovine[8], ovine[8], and porcine[37] submaxillary glands, RSL-minor differs appreciably from its major counterpart. For purposes of making a comparison of the two, the data reported in Tables I and II have been recalculated so as to be expressed in terms of mol of component per 100 mol of amino acids, and are shown in Table III. As indicated in the Table, RSL-minor has a lower relative total carbohydrate content than has RSL-major. It also contains significantly higher proportions of acidic (especially glutamic acid), basic, and aromatic amino acids, and correspondingly lower amounts of serine and threonine.

Protein–carbohydrate linkage. — The *O*-glycosyl nature of the carbohydrate–protein linkage in RSL-major was demonstrated by the alkali-catalyzed β-elimination reaction. As shown in Table IV, samples of glycoprotein treated with alkaline sodium borohydride for periods of time ranging from 5 to 10 h, and then reduced in the presence of colloidal palladium, showed substantial losses of the hydroxyamino acids serine and threonine, and corresponding increases in alanine and α-aminobutyric acid, respectively. The maximum extent of β-elimination was observed after 10 h, at which point 71% of the total seryl and 70% of the total threonyl residues had been lost. Under the conditions used, the only other amino acids that showed changes in

TABLE IV

CHANGES IN THE AMINO ACID COMPOSITION[a] OF RSL-MAJOR ASSOCIATED WITH THE ALKALINE β-ELIMINATION REACTION FOLLOWED BY REDUCTION[b]

Amino acid	*Reaction time (h)*		
	0	*5*	*10*
Threonine	40.1	18.8	11.6
α-Aminobutyric acid	0	21.0	27.8
Loss of threonine	0	21.3	28.5
Recovery (%)[c]		99	98
Serine	32.6	12.0	9.7
Alanine	21.8	42.4	43.3
Loss of serine	0	20.6	22.9
Increase of alanine	0	20.6	21.5
Recovery (%)[d]		100	94
Glycine	15.7	17.2	18.4

[a]Data are reported as mmol of amino acid per 100 g of original glycoprotein. [b]Samples were treated with 0.3M sodium borohydride in 0.1M sodium hydroxide at 45° for specified times, and then reduced according to the method of Downs *et al.*[30]. [c]Equal to α-aminobutyric acid (mmol)/loss of threonine (mmol) × 100. [d]Equal to increase of alanine (mmol)/loss of serine (mmol) × 100.

TABLE V

CHANGES IN COMPOSITION[a] OF COMPONENTS ASSOCIATED WITH THE ALKALINE β-ELIMINATION REACTION OF RSL-MAJOR

Component	*Conditions[b] of Bertolini and Pigman[29]*		*Conditions[c] of Carlson[32,33]*	
	Destruction	*Formation*	*Destruction*	*Formation*
Serine	23		21	
Threonine	29		19	
2-Amino-2-deoxy-D-galactose	44		37	
2-Amino-2-deoxy-D-glucose	17		2	
D-Galactose	10		0	
2-Amino-2-deoxy-D-galactitol		38		39
2-Amino-2-deoxy-D-glucitol		20		0

[a]The results are reported in mmol of component lost or gained per 100 g of dry glycoprotein. [b]0.3M Sodium borohydride, 0.1M sodium hydroxide, 10 h, 45°. [c]M Sodium borohydride, 0.05M sodium hydroxide, 16 h, 45°.

concentration were glycine, which increased with time and arginine, which decreased slightly.

Assay of the contents of hexosamine and hexosaminitol in the samples after 10 h of alkali treatment indicated that whereas the destruction of 2-amino-2-deoxy-D-galactose (Table V) was approximately equal to the loss of serine and threonine, its recovery as 2-amino-2-deoxy-D-galactitol was only 81% of that expected. The

glycoprotein also exhibited a loss of 2-amino-2-deoxy-D-glucose after treatment, which was accompanied by a corresponding production of 2-amino-2-deoxy-D-glucitol. These results suggest that, as with other salivary glycoproteins, the carbohydrate chains of the rat sublingual glycoprotein are attached by an *O*-glycosyl linkage of a reducing terminal 2-acetamido-2-deoxy-D-galactose residue to the hydroxyl groups of seryl and threonyl residues in the protein core. The production of 2-amino-2-deoxy-D-glucitol and the low recovery of 2-amino-2-deoxy-D-galactitol suggest that a secondary peeling-reaction may have occurred. This conclusion was confirmed by the results of the β-elimination reaction achieved under the conditions of Carlson[32,33] (Table V, column B). Although the extent of reaction is not as great as that observed at the higher alkali concentration, an excellent correlation exists between the amount of 2-acetamido-2-deoxy-D-galactose lost and that recovered as its glycitol. Also, no significant destruction of 2-acetamido-2-deoxy-D-glucose was evident.

Acid cleavage of sialic acid. — The amount of sialic acid liberated from RSL-major by hydrolysis with dilute sulfuric acid (pH 2.0, 90 min, 85°) is shown in Table VI. Ion-exchange chromatography of the hydrolyzates separated free and protein-bound sialic acid components, which were determined by the resorcinol method[12,13]. Free sialic acid was also determined after column chromatography by the Warren procedure[15].

TABLE VI

MILD ACID HYDROLYSIS OF RSL-MAJOR[a]

Compound	*Method*	
	Resorcinol	*Thiobarbituric acid*
Total sialic acid[b]	24.5	0
Free sialic acid	18.8	10.7
Protein-bound sialic acid	3.6	[c]
Recovery[d]	22.4 (91%)	[c]

[a]Samples treated with dilute sulfuric acid (pH 2.0) for 90 min at 85°. Results are expressed in terms of g of *N*-acetylneuraminic acid per 100 g of sample. [b]Determined prior to hydrolysis. [c]Not determined. [d]Equal to the sum of the free and protein-bound sialic acids.

The hydrolytic conditions used removed 85% of the original sialic acid. Attempts to increase the extent of removal by longer hydrolysis times led to a further decrease in bound sialic acid, but was accompanied by a decrease in free sialic acid, presumably as a result of acid destruction. Increase of acid concentration produced a similar result. Assay of free sialic acid by the thiobarbituric acid procedure[15] gave values which were approximately 60% lower than those obtained by the column resorcinol method.

Enzyme cleavage of sialic acid. — The results of the treatment of RSL-major with *V. cholerae* neuraminidase before and after removal of its *O*-acetyl groups are

shown in Table VII. The sialic acid residues of the intact glycoprotein were not removed by the enzyme. Removal of the *O*-acetyl groups, however, enabled a complete removal of the sialic acid residues after 24 h of enzyme treatment. The *O*-acetyl groups were removed by a 2-min exposure to alkaline hydroxylamine at room temperature. This treatment caused an average loss of 1.5 mol of *O*-acetyl residues per mol of sialic acid. Amino acid analysis of the resultant product indicated that there was also a slight loss (<5%) of serine and threonine.

TABLE VII

NEURAMINIDASE TREATMENT OF RSL-MAJOR

Determination	*Glycoproteins*		
	RSL-Major		*Hydroxylamine-treated RSL-Major*
	Sample size (mg)		
	1.472	*0.318*	*0.386*
O-Acetyl group[a]	1.6	1.6	0.1
Total *N*-acetylneuraminic acid residues[b]	25.3	25.5	24.4
Free *N*-acetylneuraminic acid residues[c]	0	0	24.1

[a]Content expressed as mol of acetyl per mol of *N*-acetylneuraminic acid. [b]Content expressed as g of anhydro *N*-acetylneuraminic acid per 100 g of glycoprotein. [c]Total sialic acid was determined for each sample by the Svennerholm procedure[13] and free sialic acid by the alkaline Ehrlich method[36].

DISCUSSION

Two sialic acid-containing mucus-glycoproteins were isolated from the rat sublingual gland. Their combined sialic acid content, as determined by the resorcinol procedure, was calculated to be 23.9 μmol/g of sublingual tissue. The sialic acid content of adult rat sublingual tissue has been investigated in a number of laboratories. Spicer and Warren[5] have reported values of 30 μmol/g by the thiobarbituric acid procedure after hydrolysis in 0.1M sulfuric acid for 1 h at 80°. Gamper *et al.*[6], with the same method but without indicating the hydrolytic parameters, found 20 μmol/g of tissue. In preliminary work, we have determined the sialic acid content of some of the glands used in this investigation by both the resorcinol and thiobarbituric acid procedures, by hydrolyzing at pH 2, for 90 min at 85°, and have found average values of 32 and 21 μmol/g of tissue, respectively. These findings, which are in good agreement with the previously reported data, indicate that the recovery of sialic acid in the isolated glycoproteins is approximately 80% of the total tissue content.

In the isolation of bovine, ovine, and porcine submaxillary mucus-glycoproteins, a final treatment of the preliminary products resulted in the separation of major and minor glycoproteins. The major materials have each been examined for

homogeneity by methods which include immunoelectrophoresis and immunodiffusion[38,39], Tiselius electrophoresis[8,37], and analytical ultracentrifugation[26], and have been shown to be free of contamination. The minor materials, however, although not as well studied, are probably heterogeneous. In unpublished work, T. Ericson (Dental School, University of Göteburg) found that the "minor" fraction from bovine submaxillary glands contained both a glycoprotein and a basic protein similar to protamines.

Because of the small amounts of rat sublingual glycoprotein available, the use of immunological methods and Tiselius electrophoresis to demonstrate homogeneity was not possible. The high molecular-weight also prohibited the use of disc-gel electrophoresis. It was, therefore, necessary to base our estimates of purity mainly on the results of analytical ultracentrifugation. On this basis, RSL-major seems to be homogeneous. RSL-minor, on the other hand, is both heterogeneous and contaminated by hydroxyapatite gel.

The chemical composition of RSL-major differs appreciably from that of other salivary mucus-glycoproteins. The carbohydrate portion, which corresponds to about 85% by weight of the intact material, contains substantial proportions of sialic acid, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, and D-galactose. Small proportions of L-fucose and D-mannose are also present. The occurrence of the latter compound is somewhat unusual for mucus glycoproteins, although a small amount has been observed in less purified preparations of porcine submaxillary mucin[40]. As with bovine sublingual[41] and canine submaxillary[42] glycoproteins, 2-acetamido-2-deoxy-D-glucose makes a substantial contribution to the total hexosamine content of both RSL-major and -minor. The rat materials differ, however, from the just-mentioned glycoproteins in that they contain more 2-acetamido-2-deoxy-D-glucose than 2-acetamido-2-deoxy-D-galactose.

The protein core of RSL-major, although present in a proportion lower than that observed for other salivary glycoproteins, shows an amino acid composition similar to that of the earlier reported products. Serine, threonine, glycine, alanine, proline, glutamic acid, and aspartic acid make up the bulk of the total amino acid content. Cysteine, tyrosine, phenylalanine, and histidine are present in only small proportions.

The nature of the sialic acid component of RSL-major was determined by its chromatographic behavior, insensitivity to the action of neuraminidase, low reactivity in the thiobarbituric acid assay, and *O*-acetyl content. The glycoprotein was found to contain 1.6 mol of *O*-acetyl groups per mol of sialic acid. This value could be interpreted as an indication of the presence of a mixture of mono-*O*- and di-*O*-acetyl-*N*-acetylneuraminic acids. Insensitivity to the action of neuraminidase indicated that one *O*-acetyl group is located at O-4 of the sialic acid molecule[43]. The thiobarbituric acid assay has been observed[43,44] to give low color-values for materials containing sialic acids that are acetylated at O-7 or -8, or that are *N*-glycolyl derivatives[15]. Since RSL-major contains very little *N*-glycolylneuraminic acid, its low reactivity suggests that the remaining *O*-acetyl group is at O-7 or O-8 in the molecule. Hence, the most

probable composition is a mixture of *N*-acetyl-4-*O*-acetyl- and *N*-acetyl-4,7(8)-di-*O*-acetylneuraminic acids.

It is evident that the presence of *N*-acetylneuraminic acid, as demonstrated chromatographically, is due to a partial *O*-deacetylation of the sialic acids in the course of hydrolysis. Schauer *et al.*[43,45] have suggested that, to avoid the rapid hydrolysis of *O*-acetyl groups, the release of sialic acid should be achieved in several steps under very mild conditions (pH 2–2.5, 1 h, 70°). Even under these conditions, substantial losses of *O*-acetyl groups occurs.

Alkali treatment of RSL-major according to the conditions of Bertolini and Pigman[29] (0.1M sodium hydroxide, 0.3M sodium borohydride, 10 h, 45°) caused a decrease in the hydroxyamino acid content of 51 mmol/g of glycoprotein, which was accompanied by an almost corresponding decrease in 2-acetamido-2-deoxy-D-galactose content. The recovery of 2-acetamido-2-deoxy-D-galactitol, however, was lower than expected. In addition, a decrease in 2-acetamido-2-deoxy-D-glucose content and the production of the corresponding glycitol was observed. These results can be interpreted as either an indication that both hexosamines are involved in the *O*-glycosyl linkage, or that the loss of 2-acetamido-2-deoxy-D-glucose is the result of a secondary peeling reaction. To clarify the situation, the β-elimination reaction was repeated under the conditions of Carlson[32,33] (0.05M sodium hydroxide, M sodium borohydride, 16 h, 45°), which have been reported to limit the extent of peeling. Under these conditions, no significant loss of 2-acetamido-2-deoxy-D-glucose content occurred. Thus, the *O*-glycosyl linkage to the protein core of RSL-major is through reducing terminal 2-acetamido-2-deoxy-D-galactosyl residues of the oligosaccharide chains. The observed peeling would be possible if 2-acetamido-2-deoxy-D-glucose is attached *via* a (1→3) linkage to the reducing terminal hexosamine[46,47].

Although the conditions of Carlson[32,33] successfully prevent peeling, the extent of β-elimination after 16 h of treatment, as determined by destruction of serine and threonine, is less than that obtained after 10 h with the procedure of Bertolini and Pigman[29]. This observation is consistent with the findings of Weber and Winzler[48] that the rate of the β-elimination reaction is dependent on the concentration of alkali rather than borohydride.

In addition to the expected decreases in proportions of hydroxyamino acids after alkali treatment of RSL-major, changes in the content of glycine and arginine were also observed. The increase in glycine, which was progressive with time of exposure to alkali, is probably due to a reverse aldol condensation of seryl or threonyl residues of the glycoprotein, as demonstrated with model compounds by Vercellotti *et al.*[49]. Decreases in arginine content have been observed previously by Downs and Pigman[50] in alkali-treated samples of bovine submaxillary glycoprotein. This loss was shown by us to arise from an alkali cleavage of arginine to ornithine, which appears as lysine under the usual conditions for amino acid analysis of protein hydrolyzates. This conversion is particularly noticeable when the alkali treatment is carried out at 45°.

As shown in Table IV, the maximum extent of the β-elimination reaction of

RSL-major was achieved after 10 h of treatment, at which point 71% of the total seryl and 70% of the total threonyl residues had been converted into their respective unsaturated derivatives. This destruction is equivalent to a net hydroxyamino acid loss of 28.5 mol/100 mol of amino acid. If we assume that this loss is an accurate assessment of the total number of *O*-glycosyl linkages per 100 amino acids, then it is possible to use the analytical data presented in Table III to deduce the average composition of the oligosaccharides present in RSL-major. For this purpose, analytical data have been recalculated in terms of mol of sugar per mol of alkali–borohydride-labile hydroxyamino acid, and are shown in Table VIII.

TABLE VIII

RELATIVE CONTENT OF CARBOHYDRATE CHAIN COMPONENTS OF RSL-MAJOR[a]

	Relative content
Sialic acid	1.7
GlcNAc[b]	1.7
GalNAc[b]	1.2
D-Galactose	1.8
L-Fucose	0.2
D-Mannose	0.3

[a]Results are expressed as mol of sugar per mol of alkali–borohydride-labile hydroxyamino acid.
[b]For abbreviations, see Table I.

The molar ratios obtained suggest an average oligosaccharide having the following structure:

$$\begin{bmatrix}(\text{Sialic Acid})_{1.7}\\(\text{Fuc})_{0.2}\end{bmatrix} \rightarrow \begin{bmatrix}(\text{Gal})_{1.8}\\(\text{Man})_{0.3}\end{bmatrix} \rightarrow \begin{bmatrix}(\text{GlcNAc})_{1.7}\\(\text{GalNAc})_{0.2}\end{bmatrix} \rightarrow \text{GalNAc}$$

The assignment of sugar residues to positions in the oligosaccharide chain was made on the following basis: (a) Since the oligosaccharides contained in RSL-major are attached to the protein core by an *O*-glycosyl linkage of 2-acetamido-2-deoxy-D-galactose to the hydroxyamino acids serine and threonine, a 1 to 1 relationship between the number of reducing terminal-residues of this sugar and the number of alkali–borohydride-labile hydroxyamino acid residues should exist. The number of residues of 2-acetamido-2-deoxy-D-galactose in excess of this amount should, therefore, occupy nonreducing terminal positions in the chain. (b) The destruction of 2-acetamido-2-deoxy-D-glucose due to secondary peeling, which was observed experimentally (Table V), suggests that this sugar occupies the second position in the oligosaccharide chain. (c) Sialic acid and L-fucose have been generally found to occupy nonreducing terminal positions in glycoprotein oligosaccharide-chains. In the case of sialic acid, this observation was confirmed experimentally by means of mild acid and enzymic hydrolysis. (d) Because of the lack of direct experimental

evidence, D-galactose and D-mannose were arbitrarily assigned to the third position in the chain.

It is evident, from the structure proposed, that the average oligosaccharide chain present in RSL-major contains approximately 7 sugar residues. Because of the marked microheterogeneity observed in the oligosaccharide derived from porcine[32] and bovine[29] submaxillary mucins, it is probable that RSL-major contains smaller and larger chains as well.

Recently, Keryer *et al.*[51] reported the isolation of mucus glycoproteins from rat submaxillary glands derived from animals of various ages and sex. The materials obtained by them from adult, male and female animals was similar to our RSL products. In our study of the sublingual glycoproteins, careful consideration was given to the possibility of contamination arising from the submaxillary gland, which is located directly adjacent to the sublingual gland.

In earlier work, we attempted to prepare the rat submaxillary glycoprotein but found that the amount of extractable, sialic acid-containing glycoproteins was too small for purification and characterization. This result is in agreement with histochemical studies that indicated that the rat submaxillary gland contains very little sialic acid. In our opinion, the products described by Keryer *et al.*[51] were derived from the sublingual gland, since no mention was made of its existence or removal.

REFERENCES

1 W. PIGMAN AND J. MOSCHERA, in J. BLANDAU AND K. MOGHISSI (Eds.), *The Biology of the Cervix*, University of Chicago Press, Chicago, 1973, pp. 143–172.
2 A. GOTTSCHALK, *Glycoproteins*, 2nd edn., Elsevier, Amsterdam, 1972.
3 R. G. SPIRO, *Ann. Rev. Biochem.*, 39 (1970) 599–637.
4 M. BERTOLINI AND F. BETTELHEIM, in W. PIGMAN AND D. HORTON (Eds.) *The Carbohydrates* Vol. IIB, Academic Press, New York, 1970, pp. 677–684.
5 S. S. SPICER AND L. WARREN, *J. Histochem. Cytochem.*, 8 (1959) 135–137.
6 C. H. GAMPER, A. A. TOCCI, A. B. HOUSSAY, H. M. CURBELO, AND J. J. DEVALLE, *J. Dent. Res.*, 49 (1970) 346–349.
7 G. QUINTARELLI, S. TSUIKI, Y. HASHIMOTO, AND W. PIGMAN, *J. Histochem. Cytochem.*, 9 (1961) 176–183.
8 G. TETTAMANTI AND W. PIGMAN, *Arch. Biochem. Biophys.*, 124 (1968) 41–50.
9 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265–275.
10 F. DOWNS AND W. PIGMAN, *Int. J. Protein Res.*, 1 (1969) 181–184.
11 G. BLIX, *Acta Chem. Scand.*, 2 (1948) 467–473.
12 L. SVENNERHOLM, *Biochim. Biophys. Acta*, 24 (1957) 604–611.
13 L. SVENNERHOLM, *Acta Chem. Scand.*, 12 (1958) 547–554.
14 S. HESTRIN, *J. Biol. Chem.*, 180 (1949) 249–261.
15 L. WARREN, *J. Biol. Chem.*, 234 (1959) 1971–1975.
16 L. SVENNERHOLM, *Acta Soc. Med. Upsal.*, 61 (1956) 75–85.
17 R. G. SPIRO, *Methods Enzymol.*, 8 (1966) 3–26.
18 E. KLENK AND G. UHLENBRUCK, *Z. Physiol. Chem.*, 307 (1957) 266–271.
19 E. SVENNERHOLM AND L. SVENNERHOLM, *Nature*, 181 (1958) 1154.
20 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature*, 166 (1950) 444–445.
21 Z. DISCHE AND A. DANILCHENKO, *Anal. Biochem.*, 21 (1967) 119.
22 Z. DISCHE AND L. B. SHETTLES, *J. Biol. Chem.*, 195 (1948) 595–603.
23 W. NIEDERMEIER, *Anal. Biochem.*, 40 (1971) 465–475.
24 C. A. ANTONOPOULOS, *Acta Chem. Scand.*, 16 (1962) 1521–1522.

25 Z. DISCHE, *J. Biol. Chem.*, 171 (1947) 725–730.
26 N. PAYZA, M. ROBERT, AND A. HERP, *Int. J. Protein Res.*, 2 (1970) 109–115.
27 J. M. CREETH AND C. G. KNIGHT, *Biochem. J.*, 105 (1967) 1135–1145.
28 W. PIGMAN, J. MOSCHERA, M. WEISS, AND G. TETTAMANTI, *Eur. J. Biochem.*, 32 (1973) 148–154.
29 M. BERTOLINI AND W. PIGMAN, *J. Biol. Chem.*, 242 (1967) 3776–3781.
30 F. DOWNS, A. HERP, J. MOSCHERA, AND W. PIGMAN, *Biochim. Biophys. Acta*, 328 (1973) 182–192.
31 K. TANAKA AND W. PIGMAN, *J. Biol. Chem.*, 240 (1965) PC1487.
32 D. M. CARLSON, *J. Biol. Chem.*, 243 (1968) 616–626.
33 J. W. MAYO AND D. M. CARLSON, *Carbohyd. Res.*, 15 (1970) 300–303.
34 A. M. BELLA, JR. AND Y. S. KIM, *J. Chromatogr.*, 51 (1970) 314–315.
35 A. S. R. DONALD, *J. Chromatogr.*, 35 (1968) 106–107.
36 D. AMINOFF, *Biochem. J.*, 81 (1961) 384–392.
37 M. DE SALEGUI AND H. PLONSKA, *Arch. Biochem. Biophys.*, 129 (1969) 49–56.
38 N. PAYZA, L. MARTINEZ, AND W. PIGMAN, *Anim. Blood Groups Biochem. Genet.*, 1 (1970) 195–206.
39 M. I. HOROWITZ, L. MARTINEZ, AND V. L. N. MURTY, *Biochim. Biophys. Acta*, 83 (1964) 305–317.
40 Y. HASHIMOTO, S. HASHIMOTO, AND W. PIGMAN, *Arch. Biochem. Biophys.*, 104 (1964) 282–291.
41 W. PIGMAN AND G. TETTAMANTI, in E. ROSSI AND E. STOLL (Eds.), *4th Int. Conf. Cystic Fibrosis Pancreas (Mucoviscidosis)*, Part II, Karger, Basel/New York, 1968, pp. 117–140.
42 C. LOMBART AND R. J. WINZLER, *Biochem. J.*, 128 (1972) 975–977.
43 V. R. SCHAUER AND H. FAILLARD, *Z. Physiol. Chem.*, 349 (1968) 961–968.
44 G. B. PAERELS AND J. SCHUT, *Biochem. J.*, 96 (1965) 787–792.
45 V. R. SCHAUER, *Angew. Chem.*, 12 (1973) 127–138.
46 R. W. JEANLOZ AND M. TRÉMÈGE, *Fed. Proc.*, 15 (1956) 282.
47 K. O. LLOYD, E. A. KABAT, AND E. LICERIO, *Biochemistry*, 7 (1968) 2976–2990.
48 P. WEBER AND R. J. WINZLER, *Arch. Biochem. Biophys.*, 129 (1969) 534–538.
49 J. R. VERCELLOTTI, R. FERNANDEZ, AND C. J. CHANG, *Carbohyd. Res.*, 5 (1967) 97–101.
50 F. DOWNS AND W. PIGMAN, *Biochemistry*, 8 (1969) 1760–1766.
51 G. KERYER, G. HERMAN, AND B. ROSSIGNOL, *Biochim. Biophys. Acta*, 297 (1973) 186–191.