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PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF A MOUSE SUBMANDIBULAR SIALOMUCIN

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ABSTRACT

A sialomucin from the mouse submandibular gland was isolated and purified by a protocol involving Sephacryl S-200 chromatography, acidic dialysis, and preparative, poly(acrylamide)-gel electrophoresis. The mucus glycoprotein was judged to be free from contaminants by analytical and sodium dodecyl sulfate–poly(acrylamide)-gel electrophoresis, isoelectric focusing, immunoelectrophoresis, and immunodiffusion when made visible by Stains-all, periodic acid–Schiff reagent, and Coomassie Blue. The carbohydrate portion constituted 81% of the weight of the mucus glycoprotein, and was composed of 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, sialic acid, D-galactose, and D-mannose. Neither L-fucose nor sulfate was detected. The aliphatic amino acids constituted 60% of the protein core. The sialomucin has an apparent mol. wt. of 140,000 by sodium dodecyl sulfate–gel electrophoresis, and a pI of 2.77–3.63 by isoelectric focusing.

INTRODUCTION

The sialoglycoproteins from ovine, bovine, and porcine submandibular glands have been isolated by a procedure that utilizes precipitation with an ammonium organic salt[1]. Attempts to apply this approach to the submandibular glands of such common laboratory animals as rats and mice have been unsuccessful[2,3], the major problem being that the concentration of sialomucin in rodent submandibular glands is relatively low compared to that in glands of other animals and in sublingual glands. The submandibular glands in mice contain about one tenth of the sialic acid per unit (wet) weight present in sublingual glands[4].

Alternatively, electrophoretic or chromatographic procedures have been employed in isolation techniques for sialoglycoproteins in rodents[3,5]. However, in each of these studies, the high content of protein in the products suggested that they were of questionable purity[2,6]. A further problem in isolation from rodents is the proximity of their sublingual and submandibular glands. Moschera and Pigman[2]

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attempted to purify mucus glycoprotein from the rat submandibular gland, but noted that the mucin was probably derived from the rat sublingual gland imbedded within the submandibular gland; they disputed the origin of the rat "submandibular"-mucus glycoprotein described by Keryer *et al.*[5], as no mention had been made of removal of the sublingual from the submandibular gland.

Our attempts to isolate mucus glycoprotein from the submandibular glands of mice by the method of Tettamanti and Pigman[1] proved unsuccessful, and we now present a new method for the isolation of a mucus glycoprotein from the mouse submandibular gland. Additionally, both an assessment of the purity of the sialoglycoprotein by a variety of criteria, and its biochemical characterization, are presented.

EXPERIMENTAL

Isolation. — Submandibular glands were obtained from 100 post-pubertal, female, Swiss–Webster mice, 80–90 days old (27–30 g). On the day of sacrifice, food was denied in the morning, although water remained available. The mice were killed by chloroform anesthesia during the early evening, and the glands were removed immediately. The submandibular was separated from the sublingual gland, washed in physiological saline at 4°, pooled, and stored at —70° until ready for use. The average, wet weight of a submandibular gland was 72 mg.

The frozen, submandibular glands were thawed, and homogenized in 0.01M Tris (pH 7.6 at 25°)–0.25M sodium chloride buffer in a Tenbroeck homogenizer (Kontes, Vineland, NJ 08360) at 4°. The homogenate was centrifuged at 50,000*g* for 45 min at 2°, and the supernatant liquor (S-50 fraction) was filtered through two layers of 130 Nitex to remove any remaining lipid.

The supernatant liquor was filtered, and the filtrate applied to a column (5 × 80 cm) of Sephacryl S-200 Superfine (Pharmacia Fine Chemicals, Piscataway, NJ 08854) equilibrated with 0.01M Tris (pH 7.6 at 25°)–0.25M sodium chloride. Fractions were eluted with the buffer at 4°, at a flow rate of 160mL/h. Fractions containing sialic acid were dialyzed overnight against 0.10M sodium acetate (pH 4.85) at 4°. The dialyzate was centrifuged at 12,000*g* for 10 min at 2°, and the clear, supernatant liquor was dialyzed at 4° against de-ionized water, and applied to a 3.75%, preparative poly(acrylamide) gel, using a Buchler Poly Prep-200 (Buchler Instruments, Inc., Fort Lee, NJ 07024) with continuous elution. The upper buffer was 25mM Tris–0.20M glycine (pH 8.3). The lower electrode-buffer and the elution buffer were three times as concentrated as the upper buffer. The eluate was collected, and the fractions containing sialic acid were determined, pooled, dialyzed against water, and centrifuged at 10,000*g* for 10 min at 4°, to remove poly(acrylamide) fragments possibly included. The supernatant liquor was collected.

Analytical methods. — After hydrolysis of samples in constant-boiling hydrochloric acid in sealed, evacuated tubes for 22 and 67 h at 110°, amino acids were analyzed in a Beckman 120B amino acid analyzer (Beckman Instruments Inc., Fullerton, CA 92634). The protein content of the purified mucin was determined by quantitating the individual amino acids. The protein content of the sample prior to preparative gel-electrophoresis was determined by the Hartree modification[7] of the method of Lowry *et al.*[8], using bovine serum albumin (BSA) as the standard.

Neutral sugars, amino sugars, and sialic acids were quantitated by g.l.c. Samples were methanolyzed in 1.5M methanolic HCl in a sealed tube[9] for 24 h at 85°. On neutralization of the acid with Ag_2CO_3, the methyl glycosides were acetylated for 6 h at room temperature. The sediment was removed by centrifugation, and the acetylated samples were dried under vacuum, overnight at 35°. Sugars were per(trimethylsilyl)ated by the addition of 3:1:9 hexamethyldisilazane–chlorotrimethylsilane–pyridine[10]. Xylose served as the internal standard. Sugar resolution was effected in a Varian 2100 gas chromatograph (Varian, Palo Alto, CA 94303) which utilized a hydrogen-flame ionization-detector. A glass, U-shaped column (2 m × 3 mm), packed with 3% of SE-30 on Gas Chrom Q, was injected with a sample at an initial temperature of 150°, and programmed at 4°/min to a final temperature of 230°. Sialic acid was additionally determined by the resorcinol method[11], and routinely determined by the thiobarbituric acid assay[12] using *N*-acetylneuraminic acid as the standard. Samples quantitated by the last method were hydrolyzed with 0.05M H_2SO_4 for 1 h at 80°.

Samples for sulfate analysis were hydrolyzed with 4M HCl for 24 h at 105°. After centrifugation at low speed, the acid was removed by evaporation under a stream of dry nitrogen. The hydrolyzate residue was dissolved in water, and the solution applied to a column of Dowex 50W-X2 (H^+) resin. Sulfate was eluted with de-ionized water, and determined by the barium chloranilate method[13].

Analytical, poly(acrylamide)-gel electrophoresis was performed in 3.75% acrylamide (pH 8.9) having an 18.75:1 cross-link ratio, by a modification of the method of Laemmli[14]. The stacking gel contained 2.5% of acrylamide (pH 6.7) having a cross-link ratio of 4:1. Electrophoresis was conducted at 3 mA per gel in 25mM Tris (pH 8.3)–0.20M glycine. Some gels additionally contained 0.1% of sodium dodecyl sulfate (SDS). Urea (3M) was also used with some SDS gels. Samples for SDS gels were incubated in 0.1% SDS, whereas those for SDS–urea gels were additionally pretreated with 7mM 2-mercaptoethanol. Gels were stained with Coomassie Blue, periodic acid–Schiff (PAS) reagent[15], or Stains-all[16]. Phosphorylase a, and polymerized BSA, served as standards for determination of the molecular weight of the mucin.

The isoelectric point was determined by using 3.75% acrylamide with 1% Pharmalyte (Pharmacia), pH 2.5–5.0. The anode solution was 0.01M iminodiacetic acid, and the cathode solution was 0.01M 2-[1-(2-hydroxyethyl)piperazin-4-yl]ethanesulfonic acid. Samples were focused for 18 h at 4° at 150 V, and an additional 1 h at 1 kV, giving a total of 3.7 kVh. Samples were also focused for 7.5 kVh, to verify that equilibrium had been achieved. The pH was directly measured with a micro-electrode at every fifth mm of gel length.

Immunoelectrophoresis was performed with S-50 antigens, and purified sialomucin, on an agarose slide. Electrophoresis occurred for 20 min at 100 V. Some electrophoretograms were immediately stained with Coomassie Blue or with Stains-all, to make the S-50 proteins and sialomucins visible. Following electrophoresis, both the control serum and the antiserum produced in rabbits against S-50 were placed in their respective troughs for immunodiffusion. The precipitates were made visible with Amido Black stain.

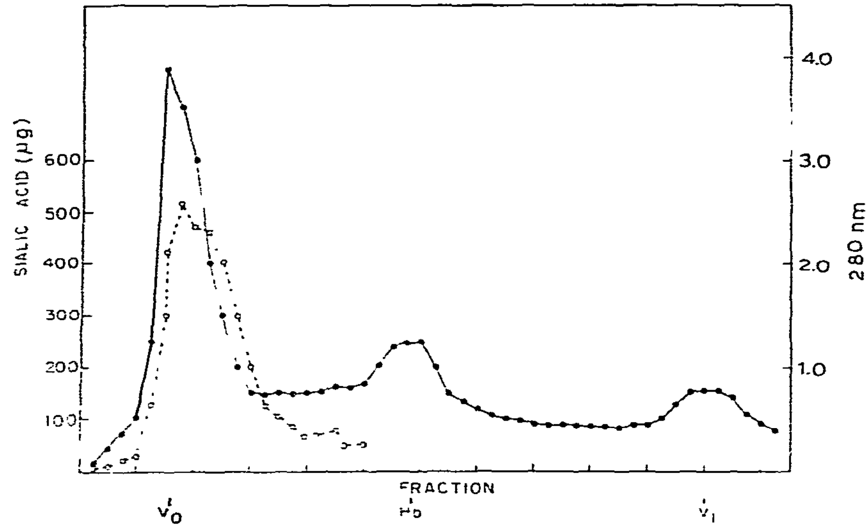

Fig. 1. Comparison of sialic acid and optical absorbance for Sephacryl S-200 chromatography. [Absorbance at 280 nm is given by the solid line, and sialic acid, which is expressed as µg per fraction, is shown by the broken line. The fractions containing sialic acid in the void volume (Vo) were those used for the sample in subsequent purification-procedures. Hemoglobin elution is indicated by Hb, and the included volume, by VI.]

TABLE I

PURIFICATION OF THE MOUSE SUBMANDIBULAR MUCIN

Mucin	*Sialic acid*[a] *(total mg)*	*Protein*[b] *(total mg)*	*Specific activity*[c]	*Purification factor*[d]	*Recovery*[e] *(%)*
Homogenate	15.15	729.00	0.021	1.0	100.0
S-50 fraction	7.82	391.00	0.020	1.0	51.7
Post-Sephacryl	3.34	32.21	0.104	4.9	22.0
Dialyzed	2.25	7.57	0.297	14.3	14.8
Preparative gel	0.94	0.58[f]	1.623	77.3	6.2

[a]Represents the values for total sialic acid by the Warren[12] method, after each step in the purification, for the glands from 100 mice. [b]Total protein, as determined by the method of Lowry *et al.*[8]. [c]The value for specific activity is expressed as the ratio of sialic acid to protein (wt : wt). [d]The values for the purification factor represent the increase in specific activity relative to the homogenate, which has been assigned a value of 1.0. [e]Represents values for percent recovery of sialic acid, subsequent to each purification step, when compared to the amount of sialic acid present in the homogenate. [f]Total protein, as determined by the amino acid analyses.

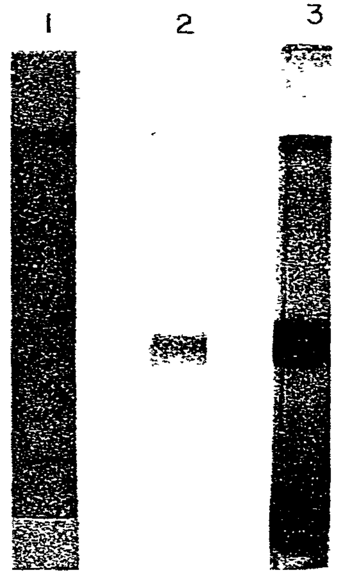

Fig. 2. Poly(acrylamide) gel of a sample after Sephacryl S-200 chromatography, and dialysis against 0.10M sodium acetate, pH 4.85. (The gel was stained with Stains-all.)

Fig. 3. Analytical poly(acrylamide) gels of electrophoretically eluted samples. [Gel 1 was stained with Coomassie Blue; Gel 2, with PAS; and Gel 3, with Stains-all. The staining material near the bottom of the Stains-all gel is Bromophenol Blue.]

Fig. 4. Poly(acrylamide) gels with the purified mucin, shown on an SDS gel (1) and an SDS-urea gel (2). [Both gels were stained with Stains-all. The lower staining region is Bromophenol Blue.]

RESULTS

Isolation. — The elution profile of the sample obtained by chromatography on Sephacryl S-200 is shown in Fig. 1. The peak contained most of the sialic acid associated with molecules of high mol. wt. Similar elution-profiles were consistently seen for other preparations. This purification step increased the specific activity almost five-fold (see Table I): however, only 22% of the original quantity of sialic acid remained. Dialysis at pH 4.85 served further to rid the preparation of nucleic acids and proteins of high molecular weight. Many purification schemes for salivary glycoproteins from other species utilize a pH of 4.7. The pH at this step is critical, however, because a pH of 4.7 may result in considerable losses of glycoprotein in rodents[2,6,17]. The specific activity was increased another five-fold by preparative gel-electrophoresis, but only 6.23% of the initial sialic acid content was retrieved.

Determination of purity. — When the sample, following dialysis at pH 4.85, was examined on analytical poly(acrylamide) gels and stained with Coomassie Blue, no staining bands resulted. Upon staining with PAS, a single, PAS-positive band appeared. These results suggest the presence of a single glycoprotein, free from contaminating proteins. When the sample was stained with Stains-all, numerous bands were revealed (see Fig. 2). Thus, the preparation still could not be considered pure, and Stains-all proved a useful indicator of purity. The gel in Fig. 2 was also sliced into 5-mm sections, and the sections were assayed for sialic acid. The broad band in the center of the gel contained 86% of the total sialic acid in the gel.

Fractions, eluted from preparative, poly(acrylamide)-gel electrophoretograms, that contained sialic acid were examined on analytical poly(acrylamide) gels. Like the dialyzed sample, stainable protein bands were not revealed, and a single, PAS-positive band resulted (see Fig. 3). Unlike the dialyzed sample, the Stains-all produced only a single, broad band and, occasionally, a faint band of material of higher mol. wt. When electrophoresed on SDS and on SDS–urea poly(acrylamide) gels (see Fig. 4), the migration rates of the mucin were identical, and no subunits were revealed. The high-mol.-wt. band seen on analytical poly(acrylamide) gels disappeared on SDS gels, suggesting that this band is not a contaminant, but a dimer[18]. Isoelectric focusing again indicated the absence of contaminants.

Immunoelectrophoresis and immunodiffusion also served as indicators of purity. Whereas the S-50 fraction showed reactivity with its corresponding antiserum, the purified mucin sample showed no bands with the antiserum made against the crude, S-50 fraction. Either no antibody was made against the mucin, or it was present

TABLE II

AMINO ACID COMPOSITION OF THE MOUSE SUBMANDIBULAR MUCIN[a]

Amino Acid	*Proportion (mol/100 mol)*[b]
Lys	5.20 ±0.09
His	0.15 ±0.09
Arg	1.53
Asp	6.75 ±0.31
Thr[c]	29.75 ±0.15
Ser[c]	5.37 ±0.08
Glu	3.37 ±0.09
Pro	14.11 ±0.45
Gly	15.95 ±0.67
Ala	9.20 ±0.39
Val	1.69 ±0.22
Met	0.15 ±0.05
Ile	2.15 ±0.10
Leu	1.99 ±0.08
Tyr	0.61 ±0.10
Phe	1.53 ±0.08
Cys[d]	0.49

[a] Values represent data from 300 mice and the mean from duplicate 22-h hydrolyses and one 67-h hydrolysis. [b] The values for standard errors of the mean are also presented. [c] The values for serine and threonine were respectively adjusted 7 and 12.9% upward, to correct for destruction during hydrolysis. [d] Determined as cysteic acid.

TABLE III

CHEMICAL COMPOSITION OF THE MOUSE SUBMANDIBULAR MUCIN[a]

Component	*Amino acid*[b] *(mol/100 mol)*	*Amino acid (wt/wt)*	*Relative wt (%)*
Protein[c]	100.0	1.00	18.9
D-Galactose[d]	53.6 ±3.15	0.87	16.5
D-Mannose[d]	19.7 ±1.02	0.32	6.1
Sialic Acid[d,e]	58.1 ±3.41	1.62	30.7
GlcNAc[d,e]	41.9 ±1.96	0.84	15.8
GalNAc[d,e]	31.6 ±2.34	0.63	11.9
L-Fucose[d]	0.0	0.0	0.0
Sulfate	0.0	0.0	0.0

[a] Data represent values from pooling the glands of 300 mice. [b] The values for standard errors of the mean are also presented. [c] Determined by amino acid analysis. [d] Determined as methyl glycosides by g.l.c. [e] Expressed as the *N*-acetyl derivative.

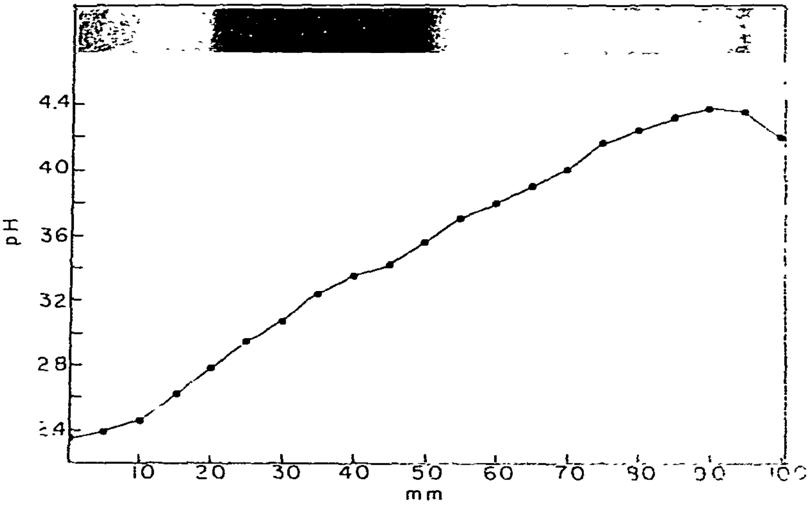

Fig. 5. Isoelectric focusing of the purified mucin shown on a 3.75% gel, with a 1% ampholyte solution, pH 2.5–5.0. [The linearity of the pH gradient of the gel is presented. The gel was stained with Stains-all. The faint band at the less acidic end of the gel always appeared, regardless of the presence of mucin.]

at a concentration below the level of detection. This experiment indicates, however, that no antigens from the crude S-50 fraction contaminated the purified, mucin preparation at detectable levels.

Amino acid and carbohydrate components. — The results of the amino acid analyses are presented in Table II, with the standard errors of the mean for each amino acid. The concentration of threonine was adjusted by assuming zero-order kinetics[19], and serine was accordingly adjusted by assuming first-order kinetics[20].

Serine and threonine constituted 35% of the protein core, whereas proline and glycine made up 30% of the core. Aromatic and sulfur-containing amino acids were present in low proportions.

G.l.c. analysis indicated that D-galactose was the principal neutral sugar present. D-Mannose was also present, but L-fucose was not detectable, indicating that it could not be present at levels higher than 0.3%. The presence of D-mannose was corroborated by colorimetric analysis[21], which also confirmed the value for the ratio of D-galactose to D-mannose provided by g.l.c. Analysis by g.l.c. showed the molar ratio of 2-acetamido-2-deoxy-D-glucose to 2-acetamido-2-deoxy-D-galactose to be 1.33:1. Good. quantitative agreement for sialic acid was obtained by g.l.c., the resorcinol method[11], and the thiobarbituric acid assay[12]. The colorimetric determination of sulfate by the barium chloranilate procedure[13] showed no sulfate (see Table III) at a sensitivity that would detect a sulfate content of 0.20%, or higher, in the mucin. The ratio sialic acid:protein (on a weight basis) was 1.62:1. Carbohydrates constituted 81% of the total weight of the glycoprotein.

By comparison of migration on SDS gels with those of standards, the apparent mol. wt. of the glycoprotein was determined to be 140.000. Isoelectric focusing (see Fig. 5) of the mucin revealed a pI of 2.77–3.63. The low isoelectric point may be due to the high concentration of sialic acid.

DISCUSSION

An electrophoretically pure. mucus glycoprotein has been isolated from the submandibular gland of the mouse. Our isolation procedure involved elimination of contaminants by a combination of techniques that are based on differences in charge and size. The apparently small mol. wt. of this mucin permitted the performance of several tests for purity that have not been applied to larger, more heterogeneous, forms isolated from other animals. The staining of disc gels with Stains-all was found especially useful in this regard, because of its capacity to detect nucleic acid contaminants. as well as other mucopolysaccharides and proteins[16]. When poly(acrylamide) gels. SDS gels, isoelectric focusing. and immunoagarose slides were stained with Stains-all. no contaminants were revealed.

The chemical composition of the mouse submandibular glycoprotein differs from those of most salivary-mucus glycoproteins, and yet it shares similarities with other rodent-mucus glycoproteins. The carbohydrate portion constitutes 81% of the weight of the molecule, and is composed of 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, sialic acid, D-galactose, and D-mannose. The proportion of carbohydrate is high compared to that in other mucus glycoproteins; however, it is similar to that in the rat sublingual gland, which is also 81% carbohydrate by weight[2]. The molar ratio of 2-acetamido-2-deoxy-D-glucose to 2-acetamido-2-deoxy-D-galactose is 1.33:1 in the mouse submandibular glycoprotein. Although this is unusual, the ratio in the rat sublingual gland is[2] 1.38:1. Neither L-fucose nor sulfate was detected, but D-mannose constituted a substantial proportion of the glycoprotein.

The absence of either L-fucose or sulfate is unusual for salivary-mucus glycoproteins, except those in rodents. The glycoprotein from hamster submandibular gland lacks L-fucose[17]. Sulfate is reportedly absent from the glycoprotein of the rat sublingual[2] and the rat submandibular gland[5]. Although the presence of D-mannose is uncommon for salivary sialomucins, it has been reported to be present both in rat sublingual[2] and mouse submandibular[3] sialomucins. Sialic acid constitutes 30% of the total weight of the mouse glycoprotein. We did not test for the presence of *O*-acetyl or *N*-glycolyl derivatives. Spicer and Warren[4] reported that no *N*-glycolyl groups are present in the mouse submandibular gland. Good agreement for sialic acid was achieved by the resorcinol[11] and the thiobarbituric acid assays[12], and by g.l.c., suggesting the absence of acylation at O-7 or O-8, in contrast to the findings for rat-sublingual mucin, which appears to contain substantial proportions of these derivatives[2].

The content of protein in the mucus glycoprotein is 19% (by weight). The value found for the aliphatic amino acid content, which constitutes 60% of the total protein, is in good agreement with that for the rat sublingual preparation[2], but differs somewhat from that for the submandibular glycoprotein prepared by Roukema *et al.*[3]. The content of protein was determined from the amino acid analysis, not by protein determination by the method of Lowry *et al.*[8], because discrepancies have been reported between these two methods[17,22]. Differences in protein content obtained by the method of Lowry *et al.*[8] may be due to the presence of sialic acid, hexosamines, or large proportions of proline and glycine, or to the absence of sulfur-containing amino acids[23].

The submandibular glycoprotein did not show separation into a major and a minor component when treated with hydroxylapatite gel. The low concentration of aromatic amino acids also indicated the absence of a minor mucin.

The apparent mol. wt. of 140,000 for the mouse submandibular mucin is small for a salivary-mucus glycoprotein. Use of SDS gel-electrophoresis of glycoproteins has been criticized, as it may lead to overestimation of the mol. wt., especially when the concentration of poly(acrylamide) is low[24]. An overestimate would further decrease the already supposedly small size of this mucus glycoprotein. Although the mucin shows considerable charge-heterogeneity by isoelectric focusing, a series of concentrations, from 12 to 0.05 μg of sialic acid, on poly(acrylamide) gels showed a narrowing of the band as the concentration was decreased. This band-narrowing with decreasing concentration indicates an unusual degree of size homogeneity for the mucin.

The mouse-submandibular sialomucin differs from most other salivary mucins by the presence of D-mannose and the higher proportion of 2-acetamido-2-deoxy-D-glucose than of 2-acetamido-2-deoxy-D-galactose. The mucin is also unique in its lack of L-fucose and sulfate, and in its low mol. wt. In most of these respects, this mucin differs from the mouse-submandibular mucin preparation obtained by Roukema *et al.*[3]. Additionally, the specific activity of sialic acid to protein is 1.62 in our preparation, whereas, in their preparation, the specific activity was 0.83. The source of these differences is not yet known; however, it could be due to developmental factors in the mice, as the difference in age between the two sample-populations spans puberty.

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