Structures and Immunochemical Properties of Oligosaccharides Isolated from Pig Submaxillary Mucins*

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SUMMARY

Two immunochemically distinct mucins have been isolated from pig submaxillary glands. The glands were combined according to the ability of aqueous extracts of these glands to inhibit hemagglutination of human type A erythrocytes. Mucin isolated from the glands containing blood group A activity is designated A+ pig submaxillary mucin (A+-PSM), while mucin isolated from the remaining glands is designated A- pig submaxillary mucin (A--PSM). The carbohydrate composition of both mucins is similar and comprises N-acetylgalactosamine, fucose, galactose, and N-glycolylneuraminic acid.

Treatment of these mucins with alkaline borohydride resulted in the release of a series of reduced oligosaccharides and the monosaccharide, 2-acetamido-2-deoxy-D-galactitol (N-acetylgalactosaminitol). Conditions are reported which give more than 90% cleavage of the sugar residues from the protein chain. The most complex oligosaccharide (designated oligosaccharide I) was a pentasaccharide, 2-acetamido-2-deoxy-D-galactopyranosyl-(1→3)-[α-1-fucopyranosyl-(1→2)]-β-D-galactopyranosyl-(1→3)-[N-glycolyneuraminyl-(2→6)]-2-acetamido-2-deoxy-D-galactitol. In addition, the following oligosaccharides were isolated (structures are given as related to oligosaccharide I): oligosaccharide II, I minus N-acetylgalactosamine; oligosaccharide III, a disaccharide N-glycolyneuraminyl-N-acetylgalactosaminitol; oligosaccharide IV, I minus N-glycolyneuraminic acid; oligosaccharide V, II minus N-glycolyneuraminic acid. N-Acetylgalactosaminitol (Fraction VI) was the only detectable monosaccharide.

Detailed knowledge of the structures of glycoproteins must necessarily precede an understanding of their biosynthesis. The glycoproteins which have blood group activity are particularly important. The early work of Bendich, Kabat, and Baeer (1) indicated that pig gastric mucosa contains blood group substances A, H, or AH. The carbohydrate composition and structure of substance A isolated from hog gastric mucosa are thought to be identical with those of the soluble human blood group A substances (2, 3). Bovine submaxillary mucin preparations contain a highly specific blood group substance (4), and human submaxillary secretions are known to contain large amounts of blood group substances (2). Thus, studies on the structure and biosynthesis of salivary mucins may also assist in understanding the synthesis of blood group substances, since these mucins often contain similar materials.

Aminoff, Morrow, and Zarafonetis (5) found that aqueous extracts of pig submaxillary glands contained substances serologically similar to the hog gastric secretions, i.e. A, H, AH, and I, which was defined as neither A nor H. These workers reported a constant ratio of fucose to hexose, and found similar sugar compositions for all serologically defined extracts. These data prompted the conclusion that the difference in immunochemical activity was not based on the absence of a particular sugar in the main oligosaccharide chain. Studies on pig serum and saliva samples (6) suggest that the interaction of alleles at the A locus with other genes is responsible for the A, O, and — phenotypes, where — is defined as neither A nor O.

Early studies of pig submaxillary mucin (7, 8) and oligosaccharides obtained from this mucin (9, 10) were carried out on pig submaxillary glands without regard for serological activity. However, recent evidence (9) suggested that a difference in pig submaxillary mucins, based on blood group activity, does exist. The present communication is a report on a detailed study of the carbohydrate moieties of two immunochemically distinct pig submaxillary mucins. Among other findings are the first proof of an oligosaccharide containing both fucose and sialic acid. The principal structural variation among the saccharides

1 Blood group O activity is assumed to correspond to the H activity reported by Aminoff et al. (5), and the phenotype “—” would be analogous to I.
isolated from the mucins is to be seen in the number of sugar residues. The structures range from a monosaccharide, N-acetylglactosaminitol, to a pentasaccharide which contains equimolar amounts of fucose, galactose, N-acetylgalactosamine, N-glycolyneraminic acid, and N-acetylgalactosaminitol.

**EXPERIMENTAL PROCEDURE**

**Materials**

Unless otherwise indicated, all materials used were of commercial origin. Sialidase was obtained from Dr. J. T. Cassidy, The University of Michigan; β-galactosidase from Dr. E. J. McGuire, The Johns Hopkins University; rabbit antihuman blood group A antisera from Dr. G. Schiffman, Hospital of the University of Pennsylvania; crystalline N-acetyleraminic acid from Dr. S. Roseman, The Johns Hopkins University; Ulex europaeus H lectin from Dr. A. Steinberg, Western Reserve University; 2,3,4-, 3,4,6-, and 2,4,6-trimethyl galactoses from Dr. P. Stoffyn, McClean Hospital, Boston; 3,4- and 4,6-dimethyl galactoses from Dr. J. K. N. Jones, Queens University, Kingston, Ontario; 2,4-dimethylgalactose from Dr. Betty Lewis, University of Minnesota. The author gratefully acknowledges the generous gifts of the indicated materials.

N-Acetylgalactosaminitol was prepared by N acetylation of chondrosine and reduction with sodium borohydride (see "Preparation of N-Acetylgalactosaminitol,") below.

**Methods**

The following substances were determined by the indicated methods: bound sialic acid by the resorcinol procedure (11) and, following sialidase treatment, by the thiobarbituric acid method (12); fucose by the method of Dische and Shettes (13); galactose by the orcinol-sulfuric acid procedure (14), corrected for fucose (19), and also by the galactose oxidase method (16); protein by the procedure of Lowry et al. (17); hexosamine by the Boas modification of the Elson-Morgan determination (18); N-acetylgalactosaminitol by a modified Morgan-Elson procedure (19); N-acetylgalactosaminitol by hydrolysis and N-acetylation with [14C]-acetic anhydride (20); reducing substances by the Park-Johnson method (21); hexoses by the anthrone procedure (22); and nitrogen by the ninhydrin method as described by Schifferman (24).

Quantitative studies of periodate consumption were made by iodometric titrations (25). Formaldehyde was measured by the chromotropic acid technique (26), and formic acid by a micrometric method (3).

N-Glycolyneraminic acid was liberated from its glycosides with sialidase according to the procedure of Caseidy, Jourdian, and Roseman (27). Treatment with β-galactosidase was carried out as outlined by Hughes and Joanloz (28). Enzymic treatment of the indicated oligosaccharides with N-acetylgalactosaminidase was performed by Dr. E. J. McGuire. The conditions reported by Kuhn, Baer, and Gauhe (29) for the hydrolysis of fucosylgalactose and fucosyl-talose (1 x H2SO4 for 180 min at 70°) were modified slightly, since incubation for 90 min at 65° was sufficient to liberate more than 80% of the fucose from some oligosaccharides. After removal of sulfate from the hydrolysate mixture with barium hydroxide, the clear supernatant fluid was N acetylated (30) to replace N-acetyl groups removed during the hydrolysis. This modified procedure will be referred to as "mild acid hydrolysis." Optical rotations were determined in a Zeiss polarimeter at 25° with water as solvent and a 559 μ light source. Radioactivity on paper chromatograms and electrophoretograms was detected with a Packard gas flow chromatogram scanner. A Packard Tri-Carb spectrophotometer was used for liquid scintillation counting and counting systems recommended by the manufacturer were used: a toluene system for counting paper strips, and a dioxane system ("DAM Cocktail 611") for aqueous solutions.

Descending paper chromatography was performed with Whatman No. 1 and with Schleicher and Schuell No. 589 Green Ribbons for characterization and isolation, respectively, of sugars. The following solvent systems were used (all in volume for volume): A, 1-butanol-pyridine-water (6:4:3); B, ethyl acetate-pyridine-water (2:1:2); C, ethanol-water-38% ammonium hydroxide (80:20:1); D, 1-butanol-acetic acid-water (4:1:5); E, 1-butanol-l-propanol-0.1 x HCl (1:2:1); F, ethyl acetate-pyridine-acetic acid-water (5:5:1:5); G, 1-butanol-ethanol-water (4:1:5); H, benzene-ethanol (4:1); I, acetonewater-38% ammonium hydroxide (250:3:1:5); J, 1-butanol-ethanol-water (10:1:2). Sugars and amino sugars on paper chromatograms were detected with periodate-benzidine (31), aniline-phosphoric acid (32), or ninhydrin (32).

High voltage electrophoresis was conducted for 45 min at 50 volts per cm on Whatman No. 3MM paper with a Gilson high voltage Electrophorator. A 1% solution of sodium tetraborate was the buffering system. Materials susceptible to periodate oxidation were detected with the periodate-benzidine technique. To preserve the blue background resulting from this procedure, it was necessary to allow the periodate to react for a maximum of 3 min and to dip the papers in the benzidine reagent while still damp. Borate was removed from electrophoretograms, if necessary, by spraying with a mixture of acetic acid-methanol (33).

Thin layer chromatography, used mainly for the separation of methylated sugars, was carried out on Silica Gel G. The plates were prepared as suggested by Brinkmann Instruments, Inc., Westbury, New York. Completely methylated sugars and other nonreducing materials were detected by spraying the plates with 55% sulfuric acid containing 0.0% potassium dichromate and charring for 30 min at 11° (34).

Charcoal-Celite column chromatography was performed with Darco G-60-Celite mixtures (1:1) prepared as described by Whistler and Durso (35). Sugars were eluted batchwise with aqueous ethanol. A Biogel P-2 column was used to desalt the oligosaccharide preparations.

The hexosamine and neutral sugar fractions of PSM were isolated as described by Sproul (36). Quantitative microprecipitin assays were performed as described by Schifferman (24).

Preparation of N-Acetylgalactosaminitol—[14C]-N-Acetylgalactosaminitol was prepared by N acetylation of chondrosine (100 mg) with [14C]-acetic anhydride under the conditions previously described (30). After acetylation was complete, one-half of the reaction mixture was removed, acidified by the addition of

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*The abbreviations used are: N-acetylgalactosaminitol, 2-acetamido-2-deoxy-p-galactitol; N-acetylhondrosinitol, β-D-glucopyranosyluronic acid-(1-3)-2-amino-2-deoxy-p-galactitol; PSM, pig submaxillary mucin; A-PSM and A'-PSM, as described in the text under "Preparation of Mucin"; N-GN and N-AN, N-glycolyl- and N-acetyleraminic acids.
Dowex 50-X8 (H+, 200 to 400 mesh), and passed through a small column of Dowex 50. The filtrate was evaporated to dryness to remove the acetic acid. The remainder of the acetylation mixture was reduced by slowly adding 200 mg of sodium borohydride with stirring in an ice bath over a period of 1 hour. Reduction was allowed to continue for an additional 2 hours, and the mixture was reduced by slowly adding 200 mg of sodium borohydride with stirring in an ice bath over a period of 1 hour. Reduction was allowed to continue for an additional 24 hours. The methylated sugars were extracted into chloroform and the chloroform was removed on a rotary evaporator. Further purification of the methylated sugars was obtained by Biogel P-2 column chromatography; the anthrone reagent was used to detect the methylated derivatives. Hydrolysis of the methylated oligosaccharides in 2 N H2SO4 for 3 hours gave the constituent methylated monosaccharides. The hydrolyzates were neutralized with Dowex 1 carbonate, and amino sugars were removed with Dowex 50-H+. The resulting solutions were evaporated to dryness at room temperature. Complete methylation of oligosaccharides was assumed if the methylated products were chromatographically homogeneous on thin layer chromatography (Solvent H) and if the expected products were obtained following hydrolysis. Trehalose and lactose gave products showing no OH band in their infrared analysis spectra.

Preparation of Mucin—The pig submaxillary mucin used in previous studies (7–10) was obtained from a pooled sample of glands not selected for blood group activity. The glands used for mucin isolation as described here were pooled according to their ability to inhibit human A-anti A hemagglutination (A+-PSM); glands which did not contain blood group A substance and consequently did not inhibit hemagglutination were designated A–PSM.

The pig submaxillary glands, obtained from a local slaughter house, were chilled in ice and excess connective tissue was removed. A small amount of tissue excised from each pair of glands was homogenized in 4 volumes of cold distilled water. The crude supernatant fluid, obtained by centrifugation for 20 min at 32,000 × g, was heated at 100°C for 10 min, cooled, and again centrifuged. The clear supernatant fluid was assayed for inhibition of the human A-anti A hemagglutination system (26), and the glands were selected accordingly. The frequency of occurrence of the A and H substances was determined for a limited number of glands (Table I). The H activity was identified by hemagglutination inhibition of human type O cells and Ulex-H antisera. The mucins (A+-PSM and A–PSM) were isolated essentially by the procedure of Hashimoto, Hashimoto, and Pigman (7) as modified by de Salegui and Pigman (38).

Isolation of Reduced Oligosaccharides—The mucins were treated with alkaline borohydride and the reduced oligosaccharides were isolated essentially as described previously (9). Approximately 1 g of mucin was incubated in 0.05 M KOH and 1.0 M sodium borohydride for 15 hours at 45°C in a final volume of 400 ml. The excess borohydride was destroyed by careful addition of 4 M acetic acid to pH 5, and the mixture was passed through a column, 4 cm × 40 cm, of Dowex 50-X8 (H+, 200 to 400 mesh). The eluate was evaporated to dryness on a rotary evaporator and

The modified procedure for purifying PSM was made available through the courtesy of Drs. M. de Salegui and W. Pigman, New York Medical College.
Carbohydrate composition of pig submaxillary mucins

The carbohydrate compositions of A+-PSM, isolated from combined H and I glands (Table I), and A-PSM, in comparison with reported values, are given. The analyses on PSM isolated in the present study are based on dry weight obtained by drying an aliquot of mucin solution to a constant weight at 100°C; no corrections were made for ash.

<table>
<thead>
<tr>
<th>Component</th>
<th>A-PSM*</th>
<th>A+-PSM*</th>
<th>PSM*</th>
<th>Glycoprotein*</th>
</tr>
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<tbody>
<tr>
<td>H glands</td>
<td>I glands</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GalNAc...</td>
<td>18.5</td>
<td>19.2</td>
<td>23.4</td>
<td>23.8</td>
</tr>
<tr>
<td>Fucose...</td>
<td>6.7</td>
<td>6.0</td>
<td>8.5</td>
<td>6.9</td>
</tr>
<tr>
<td>Galactose</td>
<td>9.0</td>
<td>8.1</td>
<td>11.5</td>
<td>12.6</td>
</tr>
<tr>
<td>N-GN......</td>
<td>15.7</td>
<td>16.1</td>
<td>14.6</td>
<td>19.8</td>
</tr>
<tr>
<td>Total carbohyd.</td>
<td>49.9</td>
<td>49.4</td>
<td>58.0</td>
<td>63.1</td>
</tr>
</tbody>
</table>

* Mucins used in the present study.
* Hashimoto, Hashimoto, and Pigman (7).
* Katzman and Eylar (8).

RESULTS AND DISCUSSION

Two immunochemically distinct mucins (A+-PSM and A-PSM) have been isolated from pig submaxillary gland extracts by methods previously reported (7, 38). The extracts were first treated at pH 4.5 and the highly charged polyanionic mucins were then precipitated as an insoluble complex with cetylpyridinium chloride. The resulting clot-like material, dissolved by adding 0.9% NaCl solution, was further purified by ethanol fractionation. Although the isolated mucins were monodisperse in the ultracentrifuge, no claim can be made for “homogeneity” or “purity” of the mucin samples. The carbohydrate compositions of mucins used in this study (Table II) are approximately the same as reported for PSM by Hashimoto, Hashimoto, and Pigman (7). Although purified A+-PSM contains more GalNAc than A-PSM, it would be indeed difficult, if not impossible, to determine blood group activity of an individual gland based on its gross sugar composition. Results based on analyses of this type undoubtedly prompted Aminoff et al. (5) to conclude that differences in the sugar chains did not exist among the serologically different mucins.

Studies of the carbohydrate structures of glycoproteins are facilitated by quantitative release of the sugar chains with a minimum of degradation. Other workers, using alkali concentrations similar to that reported here (see “Methods”) and incubating for 5 to 7 days at room temperature to release the sugar residues from mucins (10, 39) and blood group substances (3), have experienced low yields and reported degradation of the...
TABLE III

Molar ratios and sugar recoveries from alkaline borohydride-treated A+-PSM

See “Preparation of oligosaccharides” for details.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>N-GN^a</th>
<th>Fucose^b</th>
<th>Galactose^c</th>
<th>GalNAc^d</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol/g mucin</td>
<td>%</td>
<td>µmol/g mucin</td>
<td>%</td>
<td>µmol/g mucin</td>
</tr>
<tr>
<td>A+-PSM</td>
<td>400</td>
<td>100</td>
<td>290</td>
<td>100</td>
<td>840</td>
</tr>
<tr>
<td>Dowex 50-H+ eluate</td>
<td>375</td>
<td>94</td>
<td>255</td>
<td>90</td>
<td>225</td>
</tr>
<tr>
<td>Dowex 1-Cl- water eluate</td>
<td>250</td>
<td>62</td>
<td>100</td>
<td>39</td>
<td>98</td>
</tr>
<tr>
<td>Dowex 1-Cl- NaCl eluate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^a Molar ratio for sugar in this preparation, 0.96.
^b Molar ratio for sugar in this preparation, 0.63.
^c Molar ratio for sugar in this preparation, 0.77.
^d Molar ratio for sugar in this preparation, 2.00.
^e Percentage recovery of constituents, based on colorimetric analysis.

Fig. 3. Studies on alkaline borohydride conditions for cleavage of the carbohydrate to protein linkage. A+-PSM was incubated at 25° (⊙, □, △) and at 45° (●, ■, ▲) at concentrations described in the text. The alkaline conditions were 0.05 N KOH (△, ▲), 1 M sodium borohydride (○, ●), and 0.05 N KOH plus 1 M sodium borohydride (□, ■). The incubation mixtures were neutralized at the indicated time intervals and dialyzed. The contents of the dialysis bags were assayed for N-GN by the resorcinol procedure. Controls neutralized at zero time were included.

Sugar Recoveries

Molar ratios and recoveries of the constituent carbohydrate moieties of A+-PSM are given in Table III. High recoveries of sialic acid and fucose with a complete loss of hexosamine were obtained, following alkaline borohydride treatment and Dowex 50-H+ column chromatography. Hydrolysis of A+-PSM gave the same results, except that the recoveries of GalNAc varied from 30 to 40%. Galactose recoveries ranged from 70 to 85%, but there were no detectable galactitol or unsaturated compounds as in oligosaccharides from blood group substances (3) and from PSM as reported by Katzman and Eylar (39). Chromatography on Dowex 1-Cl- resulted in a 20 to 25% loss of sialic acid-containing oligosaccharides and a 10 to 20% loss of neutral oligosaccharides. Chromatography on charcoal-Celite or Biogel P-2 gave better than 90% recovery of sugars.

Characterization of Saccharides

All saccharide fractions were homogeneous in five chromatographic solvent systems and on electrophoresis in borate buffer. Oligosaccharides II, III, V, and VI were isolated from A+-PSM; this preparation did not contain oligosaccharides I and IV. A+-PSM contained the oligosaccharides found in A+-PSM and, in addition, oligosaccharides I and IV. The proposed structures are shown in Fig. 4. Oligosaccharide I, a pentasaccharide, contains equimolar quantities of GalNAc, galactose, fucose, N-GN, and N-acetylgalactosaminitol. The remaining oligosaccharides lack one or more of these sugar residues, but all contain N-acetylgalactosaminitol. The monosaccharides were isolated from A+-PSM; thus the galactosamine fraction should reflect the configuration of the amino sugar attached to the protein (see Fig. 4), since this is the only hexosamine moiety present. Fucose and galactose were the only detectable neutral sugars. The fucose, isolated as a syrup, had a specific rotation of −73° (reported value, −76° (40)). A preparation of the crystalline fucose phenylhydrazone (40) melted at 169–172°; the reported value is 170–172°. A mixture of this material with authentic fucose phenylhydrazone had a melting point of 169–171°. Galactose and galactosamine both occur in the β configuration, as shown by their rates of reaction with galactose oxidase as compared with standard compounds. The galactosamine and galactose isolated from PSM gave 73% and 98%, respectively, of the color obtained with standard galactose (16).
Neutral Fractions

**Fraction VI**—This fraction, not bound by Dowex 1-Cl⁻, was eluted from charcoal-Celite with 5% ethanol. The material was identical with N-acetylglactosaminol in Solvents A, B, and F and in borate electrophoresis. After crystallization from ethanol the material melted at 174–175° (uncorrected). Crystalline N-acetylglactosaminol, prepared by the reduction of GalNAc with sodium borohydride, melted at 174–175°. Treatment with periodate, followed by reduction with borohydride, converted both Fraction VI and N-acetylglactosaminol into products the \( R_F \) values of which were identical with that of N-acetylsorinol in three solvent systems (A, B, and D). The N-acetyl content of Fraction VI was 12.1% (theoretical, 11.6%).

**Oligosaccharide V**—Mild acid hydrolysis liberated 89% of the fucose from oligosaccharide V, as followed by the increase in reducing substances (Fig. 5), with no detectable release of galactose or N-acetylglactosaminol. Fucose was separated from the galactosyl-N-acetylglactosaminol moiety by charcoal-Celite chromatography. The disaccharide had a specific rotation of \(-48°\) (Table IV), and was cleaved by \( \beta \)-galactosidase. The high negative rotation of the trisaccharide (\(-114.7°\), Table IV) suggests that fucose is bound by an \( \alpha \) linkage (42). The specific rotation of oligosaccharide V is close to those of similar oligosaccharides isolated from blood group H substance (\(-111°\) and \(-116°\) (3)).

The galactose of oligosaccharide V is substituted at carbon atom 2, as shown by the facts that (a) the trisaccharide is active with galactose oxidase (16), and (b) methylation gave 3,4,6-trimethyl galactose (Table VI, below).

Methylation and acid hydrolysis of the disaccharide, galactosyl-N-acetylglactosaminol, gave 2,3,4,6-tetramethyl galactose (Table VI, below) and a product that was removed by Dowex 50-H⁺. Elution of this cationic material and treatment with periodate in the formation of reducing substances, which suggests that galactose had been linked to C-3 of N-acetylglactosaminol. Treatment of both the disaccharide galactosyl-N-acetylglactosaminol and N-acetylgalactosaminol with periodate, followed in succession by borohydride reduction, acid hydrolysis, and N-acetylation with \( { }^{14} \)C-acetic anhydride, gave identical \( { }^{14} \)C-products (Fig. 6). N-Acetylgalactosaminol, the product arising from N-acetylgalactosaminol, does not migrate with the above conditions and is not detectable by the periodate-benzidine technique. The absence of radioactivity at the origin eliminates the presence of this 3-carbon fragment. These data suggest that galactose is linked to C-3 of N-acetylglactosaminol and not to C-4 as suggested by Katzman and Eylar (10). The results of periodate oxidation experiments (Table V) provide additional evidence for the proposed structure of oligosaccharide V.

**Oligosaccharide IV**—Mild acid hydrolysis (180 min) released more than 80% of the fucose from oligosaccharide IV with concomitant formation of a trisaccharide which contained GalNAc, galactose, and N-acetylglactosaminol (Table IV). The galactose residues of both the parent oligosaccharide and the resulting trisaccharide were resistant to periodate oxidation, indicating that the galactose was substituted at C-3. Methyla-

4The N-acetyl analyses were performed by Crobaugh Laboratories, Cleveland.
TABLE IV
Yields, analyses, and specific rotations of oligosaccharide fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Yield* (mg/g mucin)</th>
<th>A-PSM</th>
<th>A'-PSM</th>
<th>Galactose</th>
<th>Fucose</th>
<th>GalNAc</th>
<th>N-GN</th>
<th>N-Acetyl-galactosaminotol</th>
<th>[(\Delta^\circ)]D</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0-40</td>
<td>1.06</td>
<td>0.85</td>
<td>0.86</td>
<td>1.00</td>
<td>0.94</td>
<td>99.7</td>
<td>+6.9*</td>
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<tr>
<td>II</td>
<td>48</td>
<td>1.06</td>
<td>0.52</td>
<td>1.00</td>
<td>0.91</td>
<td>94.5</td>
<td>-10.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>40</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.03</td>
<td>101.0</td>
<td>114.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>44</td>
<td>1.00</td>
<td>0.82</td>
<td>0.81</td>
<td>1.05</td>
<td>103.2</td>
<td>19.6</td>
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</tr>
<tr>
<td>V</td>
<td>27</td>
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<td>1.00</td>
<td>1.00</td>
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<td>101.0</td>
<td>114.7</td>
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<tr>
<td>VI</td>
<td>-</td>
<td>1.00</td>
<td>0.83</td>
<td>0.91</td>
<td>0.91</td>
<td>91.0</td>
<td>19.8</td>
<td>+19.8</td>
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<table>
<thead>
<tr>
<th>Fraction</th>
<th>Yield* (mg/g mucin)</th>
<th>A-PSM</th>
<th>A'-PSM</th>
<th>Galactose</th>
<th>Fucose</th>
<th>GalNAc</th>
<th>N-GN</th>
<th>N-Acetyl-galactosaminotol</th>
<th>[(\Delta^\circ)]D</th>
</tr>
</thead>
<tbody>
<tr>
<td>I minus N-GN</td>
<td>1.00</td>
<td>0.83</td>
<td>0.91</td>
<td>1.09</td>
<td>0.91</td>
<td>91.0</td>
<td>19.8</td>
<td>+19.8</td>
<td></td>
</tr>
<tr>
<td>II minus N-GN</td>
<td>1.00</td>
<td>0.83</td>
<td>0.91</td>
<td>1.09</td>
<td>0.91</td>
<td>91.0</td>
<td>19.8</td>
<td>+19.8</td>
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</tr>
<tr>
<td>II-a</td>
<td>1.00</td>
<td>0.83</td>
<td>0.91</td>
<td>1.09</td>
<td>0.91</td>
<td>91.0</td>
<td>19.8</td>
<td>+19.8</td>
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</tr>
<tr>
<td>II-b</td>
<td>1.00</td>
<td>0.83</td>
<td>0.91</td>
<td>1.09</td>
<td>0.91</td>
<td>91.0</td>
<td>19.8</td>
<td>+19.8</td>
<td></td>
</tr>
<tr>
<td>Fraction IV minus fucose</td>
<td>1.00</td>
<td>0.16</td>
<td>0.89</td>
<td></td>
<td>0.94</td>
<td>48</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction V minus fucose</td>
<td>1.00</td>
<td>&lt;0.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Yields of oligosaccharides are based on dry weights of materials precipitated with acetone.

b Calculated weight of oligosaccharide (based on analytical values) \(\times 100 = \text{percentage of dry weight}\).

c, not tested.

d Sialidase treated to remove N-GN.

e Fucose removed by mild acid hydrolysis.

TABLE V
Results of periodate oxidations

Periodate uptake and formaldehyde and formic acid formation were measured after a 24-hour incubation period.

<table>
<thead>
<tr>
<th>Oligosaccharide</th>
<th>Theoretical</th>
<th>Actual</th>
<th>Theoretical</th>
<th>Actual</th>
<th>Formic acid formed*</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>6.00</td>
<td>5.82</td>
<td>1.00</td>
<td>1.08</td>
<td>2.00</td>
</tr>
<tr>
<td>III</td>
<td>4.00</td>
<td>4.30</td>
<td>1.00</td>
<td>0.96</td>
<td>3.00</td>
</tr>
<tr>
<td>IV</td>
<td>5.00</td>
<td>5.10</td>
<td>1.00</td>
<td>0.93</td>
<td>2.00</td>
</tr>
<tr>
<td>V</td>
<td>5.00</td>
<td>4.90</td>
<td>1.00</td>
<td>0.93</td>
<td>2.00</td>
</tr>
<tr>
<td>Mannitol standard</td>
<td>5.00</td>
<td>5.30</td>
<td>2.00</td>
<td>1.92</td>
<td>4.00</td>
</tr>
</tbody>
</table>

* Oligosaccharides IV and V gave consistently low values (00 to 65% of theoretical) for periodate consumption and formic acid formation after incubation in water at 4°C.

Fig. 6. Identification of periodate oxidation products of substituted N-acetylgalactosaminotol. Mixtures containing A, N-acetylgalactosaminotol, 1.5 \(\mu\)moles, or B, galactosyl-N-acetylgalactosaminotol, 1.3 \(\mu\)moles, sodium acetate, pH 4.5, 12 \(\mu\)moles and NaIO4, 40 \(\mu\)moles, in a final volume of 0.06 ml, were incubated for 12 hours at 4°C in the dark. The reaction was terminated by adding 1 ml of 1 M NaBH4 and the mixture was allowed to stand at room temperature for 2 hours with intermittent mixing. Excess borohydride was destroyed by adding 1 M acetic acid and the solution was applied to a column of Dowex 50-H+ in the dark. The eluate, evaporated to dryness, was treated with methyl alcohol to remove boric acid. N-Acetylgalactosaminotol (1.1 \(\mu\) mole) was carried through the remaining procedure, together with the oxidized samples. Following hydrolysis in 1 M HCl for 5 hours, all samples were evaporated to dryness and N-acetylated with \(^{14}C\)-acetic anhydride. The \(^{14}C\)-N-acetylated derivatives and the indicated standards were separated by electrophoresis in borate buffer. Radioactive areas were detected by strip scanning; areas reacting with periodate-benzidine are cross-hatched. The \(^{14}C\)-products from galactosyl-N-acetylgalactosaminotol and N-acetylgalactosaminotol were also identical when chromatographed in Solvents A, D, and G. The remaining oligosaccharides (Table IV), except oligosaccharide III and Fraction VI, gave the same \(^{14}C\)-products when exposed to the above conditions.

**Sialic Acid-containing Fractions**

Oligosaccharide III—Oligosaccharide III is similar to the disaccharide, N-acetyleneuraminyl-(2→6)-N-acetylgalactosamin-
itol, isolated from sheep submaxillary mucin (43, 44), except that it contains N-GN instead of N-AN. Treatment with sialidase yielded only N-GN (95%) and N-acetylgalactosaminitol. Periodate oxidation followed by borohydride reduction gave two components: one which was absorbed by Dowex 1-Cl\(^{-}\) and which contained the sialic acid portion, and the other a neutral fragment. Hydrolysis of the neutral material in 4\(\times\)HCl for 2 hours at 100\({}^\circ\) yielded serinol, as identified by borate electrophoresis and chromatography on borated paper in Solvents A, B, and D. Hydrolysis of the sialic acid-containing material (1\(\times\)H\(\text{SO}_4\), 30 min, 100\({}^\circ\)) released ethylene glycol, as detected by paper chromatography in Solvents A and D. These data show that oligosaccharide III contains the same linkage as reported for similar disaccharides from other submaxillary mucins in which sialic acid is linked to C-6 of N-acetylgalactosamine (45).

**Oligosaccharide II**—The fraction designated oligosaccharide II is a mixture of two oligosaccharides (9). Attempts to separate these compounds prior to sialidase treatment were unsuccessful. Sialidase cleaved 89% of the N-GN from oligosaccharide II. Two neutral oligosaccharides, II-a and II-b, were then isolated. Oligosaccharide II-a contained fucose, galactose, and N-acetylgalactosaminitol (Table IV), and had the properties of oligosaccharide V. Oligosaccharide II-b contained only galactose and N-acetylgalactosaminitol (Table IV), and was identical with the disaccharide isolated from oligosaccharide V following mild acid hydrolysis. The relative amounts of oligosaccharides II-a and II-b varied with different preparations of PSM.

**Oligosaccharide I**—The most complex oligosaccharide isolated was the pentasaccharide, oligosaccharide I. Sialidase treatment gave N-GN (91%) and a product which was identical with oligosaccharide IV. Treatment of 0.08 pmole of oligosaccharide I with periodate followed by reduction with borohydride gave two compounds. The anionic fragment, removed by Dowex 1-Cl\(^{-}\), was eluted with 0.1\(\times\)NaCl and contained 0.063 pmole of sialic acid, as determined by the resorcinol procedure. Galactose (0.077 pmole), measured by the anthrone method and, following hydrolysis, with galactose oxidase, was quantitatively eluted from the resin with water. Acid hydrolysis of this latter material and N-acetylation with \(\text{N-acetyl}\) gave two radioactive compounds which were chromatographically identical with the products prepared in the same manner from oligosaccharide IV. These data support the proposed 1\(\rightarrow\)3 linkage of galactose to N-acetylgalactosaminitol and suggest that N-GN is glycosidically linked to C-6 of N-acetylgalactosaminitol, as in oligosaccharide III.

### Chromatographic identification of methylated sugars

The methylated oligosaccharides were acid hydrolyzed, de-ionized, and chromatographed on thin layer chromatographic plates and paper.

<table>
<thead>
<tr>
<th>Methylated derivative</th>
<th>Solvent system H</th>
<th>Solvent system I</th>
<th>Solvent system G</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligosaccharide IV</td>
<td>0.30</td>
<td>0.86</td>
<td>0.90</td>
</tr>
<tr>
<td>Oligosaccharide V</td>
<td>0.48</td>
<td>0.87</td>
<td>0.66</td>
</tr>
<tr>
<td>Oligosaccharide I minus N-GN</td>
<td>0.30</td>
<td>0.86</td>
<td>0.53</td>
</tr>
<tr>
<td>Oligosaccharide V minus fucose</td>
<td>0.87</td>
<td>0.84</td>
<td>0.90</td>
</tr>
<tr>
<td>2,3,4,6-Tetramethyl galactose</td>
<td>0.88</td>
<td>0.84</td>
<td>1.00</td>
</tr>
<tr>
<td>2,3,4-Trimethyl fucose</td>
<td>0.87</td>
<td>0.90</td>
<td>1.00</td>
</tr>
<tr>
<td>Tridimethyl galactoses</td>
<td>0.47</td>
<td>0.67</td>
<td>0.76</td>
</tr>
<tr>
<td>2,3,6-</td>
<td>0.67</td>
<td>0.76</td>
<td>0.76</td>
</tr>
<tr>
<td>3,4,6-</td>
<td>0.67</td>
<td>0.76</td>
<td>0.76</td>
</tr>
<tr>
<td>Dimethyl galactoses</td>
<td>0.30</td>
<td>0.58</td>
<td>0.62</td>
</tr>
<tr>
<td>2,4-</td>
<td>0.58</td>
<td>0.62</td>
<td>0.57</td>
</tr>
<tr>
<td>3,4-</td>
<td>0.58</td>
<td>0.62</td>
<td>0.57</td>
</tr>
<tr>
<td>4,6-</td>
<td>0.58</td>
<td>0.62</td>
<td>0.57</td>
</tr>
</tbody>
</table>

\(^a R_{TMG}, R_{Triamethylgalactose}\)

\(^b R_{TFM}, R_{Trimethylfucose}\)

\(^c 2,3,4\)-Trimethyl galactose does not migrate under the conditions used for borate electrophoresis; 3,4,6-trimethyl galactose and the indicated derivatives from oligosaccharides migrate toward the anode.

**Periodate Oxidation Studies**

Periodate oxidation studies, performed in buffered solutions at pH 4.5, resulted in periodate consumption and formaldehyde formation data which are in agreement with the proposed
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Fig. 9. Inhibition of the precipitin reaction by oligosaccharides. All incubations were carried out at 4° in 0.1 ml of A antiserum, 5 μg of A+-PSM, and the indicated amounts of oligosaccharides, in a total volume of 0.3 ml.

Fig. 10. Thin layer chromatography of methylated standards and oligosaccharides (Solvent H). ChonOHNAc, N-acetylchondrosinitol; (2X) and (3X) indicate the number of methylation treatments for oligosaccharide IV (see "Methods").

oligosaccharide structures. The periodate oxidation curve for oligosaccharide IV is shown in Fig. 7. Data on periodate consumption and formic acid and formaldehyde formation are given in Table V. Formic acid determinations were performed in water and consistently gave results of 60 to 65% of theoretical; this was in agreement with periodate consumption data obtained under the same conditions.

Quantitative Precipitin Assays

Rabbit antiserum to human type A red cell stroma precipitated A+-PSM, but not A-PSM (Fig. 8). Oligosaccharides I and IV inhibited the reaction of A antiserum with A+-PSM, whereas oligosaccharides II, III, V, and VI were inactive (Fig. 9). These data support the proposed structures for oligosaccharides I and IV, which contain the same antigenic sugar determinant groupings as blood group A substance (3).

Methylation Studies

Standards of L-fucose, trehalose, N-acetylcchondrosinitol, oligosaccharide V, and oligosaccharide V minus fucose appear to be completely methylated by the procedure described (Fig. 10). Oligosaccharide IV required three treatments to achieve essentially complete methylation. Chromatographic identification of the constituent methylated monosaccharides is given in Table VI.

Hydrolysis of the galactose and fucose methyl ethers with boron tribromide in dichloromethane (45) resulted in the release of galactose and fucose, respectively, as shown by paper electrophoresis in borate buffer and paper chromatography with Solvent Systems A and J.

DISCUSSION

Studies on the carbohydrate composition of purified PSM (7-9) indicate that N-GalNAc, GalNAc, galactose, and fucose account for almost the entire carbohydrate content. Traces of N-AN, mannose, and GluNAc are found in some fractions, but these components may still represent a small contamination with plasma glycoproteins. Many problems are involved in demonstrating the purity of isolated mucins. Highly charged polymers, such as these, produce viscous solutions which do not adapt readily to the usual physical methods of determining purity and homogeneity (46).

PSM, isolated by different methods, has been shown to be monodisperse in the ultracentrifuge (7, 8), electrophoretically homogeneous (7, 8), and homogeneous by polyacrylamide gel electrophoresis and immunoelectrophoresis (8). The present investigation shows that at least two immunochemically different glycoproteins are present in pig submaxillary glands. Thus, it is difficult to define a "homogeneous" polymer of this type.

Physical and chemical studies on glycoproteins purified from pooled pig submaxillary glands by different procedures (7, 8) have some striking differences. This is particularly true for glycoprotein II isolated by DEAE-cellulose-Celite column chromatography (8). High salt concentration (5 N NaCl) failed to elute this tightly adsorbed material. However, 5 N NaCl combined with a gradient of 0.05 N NaOH resulted in the elution of glycoprotein II. This material was found to be of relatively low molecular weight (180,000) and exhibited a low intrinsic viscosity (1.32 dl gm⁻¹), as compared with previous values for PSM of 810,000 and 6.7 dl gm⁻¹, respectively (7). These differences were attributed to dissociation of PSM on DEAE-cellulose under "neutral" conditions and "moderate" ionic strength (8). Further studies are required, but glycoprotein II may be the result of degradation of the mucin molecule and not a discrete glycoprotein fraction.

The products of alkaline treatment of mucopolysaccharides, mucins, and blood group substances have been studied extensively (47). The mechanism whereby the sugar moiety is released is now commonly accepted as a β elimination reaction involving glycosidic linkages to the hydroxyl groups of serine or threonine or both. While these linkages have been well defined in sheep and bovine submaxillary mucins (47), the carbohydrate to protein linkage in PSM remains to be studied. The release of...
N-acetylgalactosaminitol by alkaline borohydride indicates that the linkage in PSM is the same as that found in other mucins. We described a series of partially characterized oligosaccharides in a preliminary report (9), including the trisaccharide, fucosyl-α-galactosyl-N-acetylgalactosaminitol (oligosaccharide V). Subsequently, Katzman and Eylar (10) reported the structure of a neutral trisaccharide isolated from PSM to be α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-N-acetyl-D-galactosaminitol. Sufficient data were presented to define the linkage of fucose to GluNAc (possibly analogous to the A substance (48), in which a second fucose residue, attached to GluNAc, is encountered during alkaline borohydride treatment, but these workers failed to provide evidence for the proposed anomeric configurations or the D- or L-sugar configurations. Results of the present study provide no evidence for a 1→4 linkage of galactose to N-acetylgalactosaminitol in the oligosaccharides isolated from PSM. Instead the data indicate only a 1→3 linkage. However, the existence of a 1→4 linkage in an oligosaccharide fraction of PSM cannot be completely excluded, since the studies by Katzman and Eylar were performed on a trisaccharide that accounted for only 0.1 to 0.2% of the total carbohydrate in the mucin.

The structural complexity of blood group substances A, B, and H must be appreciated. Considerable degradation of the oligosaccharide chain is encountered during alkaline borohydride treatment, with the formation of galactitol and α-hexene-1,2,5,6-tetrol, an unsaturated galactitol derivative (3). Although different conditions were used, neither of these compounds was isolated from A and H blood group substances. They are, respectively, A_2R, 0.52 and H_2R, 0.75 (3).

Blood group A activity of oligosaccharide I increases following removal of the sialic acid residue. This result agrees with studies on the inhibition of A-anti A precipitation by oligosaccharides isolated from blood group A substance (48), in which a second fucose residue, attached to GluNAc (possibly analogous to the N-acetylgalactosaminitol residue in oligosaccharide I), decreased the inhibitory ability. Also, the release of an ionic component may influence the relative serological activity of oligosaccharide I.

Preliminary experiments in this laboratory suggest that mucins isolated from H and I type glands contain the same oligosaccharide chains. If this is so, then the immunchemical difference may be the result of variation in the relative amounts of the different oligosaccharides.

Studies are now in progress, with the precipitin technique, to attempt to show that the oligosaccharide moieties all reside on the same protein chain.

Acknowledgments—The author wishes to thank Dr. G. Schiffman for his assistance in the microprecipitin assays, Dr. E. McGuire for treating oligosaccharide IV with N-acetylgalactosaminidase, Dr. P. Stoffyn for suggesting Solvent I for the separation of trimethylgalactosyl derivatives, Mr. T. Saari for assaying the pig submaxillary gland extracts for blood group activity, and Dr. Judith Ramseyer for assisting in the infrared spectral analyses.

REFERENCES

43. PIGMAN, W., AND GOTTSCALK, A., IN A. GOTTSCALK (Editor), Glycoproteins—their composition, structure and function,


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Don M. Carlson and With the technical assistance of Charles Blackwell


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