THE ISOLATION AND CHARACTERIZATION OF A TRISACCHARIDE FROM PORCINE SUBMAXILLARY GLYCOPROTEINS*

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The glycoproteins of mucus secretions have been studied intensively in the past twenty years. The structure of the carbohydrate moieties in these glycoproteins, however, is generally unknown. Indeed, except for the disaccharide sialyl-(1→ 6)-N-acetylgalactosamine obtained from bovine and ovine submaxillary glycoproteins (Gottschalk, 1959), no other oligosaccharide has been isolated from mammalian submaxillary glycoproteins. In this paper we describe the isolation of the trisaccharide α-L-fucopyranosyl-(1→ 2)-β-D-galactopyranosyl-(1→ 4)-N-acetyl-D-galactosaminitol obtained by alkaline-reductive cleavage (Tanaka and Pigman, 1965) from three porcine glycoprotein fractions. This is the first neutral oligosaccharide to be isolated from submaxillary glycoproteins.

Materials and Methods. Porcine submaxillary mucin (PSM) was prepared by a modification of the procedure described by Hashimoto et al (1964). The Cetavlon-mucin clot was obtained from an undialyzed aqueous extract of the glands. The clot was dissolved in 0.15 M NaCl and CM-Sephadex added to remove the Cetavlon. The solution was then dialyzed and lyophilized. The product appeared to be similar, both physically and chemically, to the PSM prepared by Hashimoto et al (1964). The PSM was fractionated on DEAE-cellulose

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with NaCl gradient elution. One fraction (GP I) was eluted with approximately 0.1 N NaCl; the second fraction (GP II) eluted with 5 N NaCl. GP I contained 55% protein and 45% carbohydrate; GP II contained 40% and 60%, respectively.

The solvent systems used for paper chromatography were the following: Solvent A, 1-butanol:acetic acid:water (4:1:5); Solvent B, 1-butanol:ethanol: water (4:1:5); Solvent C, 1-butanol:pyridine:water (6:4:3); Solvent D, water saturated 1-butanol:ethanol:water:ammonia (40:10:49:1); Solvent E, water saturated 1-butanol; and Solvent F, 2,4,6-collidine:ethyl acetate:water (2:5:5) as described by Lenz and Holmberg (1956). Reducing and non-reducing sugars were detected by the alkaline silver nitrate procedure. Reducing sugars were detected with the benzidine reagent. We are indebted to Professor R. Kuhn for samples of 2'fucosyllactose and 3,4,6-tri-O-methylgalactose and Dr. B. A. Lewis for samples of 2,3,4- and 2,4,6-tri-O-methylgalactose. D-Threose was a gift from Dr. Arvin Fluharty.

Isolation. PSM (23.4 g) was dissolved in 400 ml of 0.3 N sodium borohydride in 0.1 N NaOH, covered with toluene (0.1 ml), and kept at 28° for 120 hrs. The reaction mixture was neutralized with dilute acetic acid, deionized on a mixed-bed resin (Amberlite MB-3), and lyophilized (yield, 13.8 g). This material was dissolved at room temperature in 100 ml of 75% ethanol and the insoluble material removed by centrifugation. The supernatant, combined with the washings from the precipitate, were taken to dryness (yield, 2.85 g). This product was chromatographed on Whatman 3MM paper with Solvent A for 48 hrs. Guide strips were cut from each sheet and stained, revealing nine bands with R_f's (with respect to N-acetylgalactosaminitol) of 0.15, 0.25, 0.35, 0.49, 0.64, 0.73, 1.00, 1.44, and 1.58. Identical qualitative results were obtained with GP I and GP II when treated in the same manner as described above.

Purification. The material with R_f 0.49 was eluted from the sheets (yield, 408 mg) and upon chromatography in Solvent B was found to contain Bands 5, 6, and 7 as minor contaminants. A portion of the material (127.5 mg) was purified further by charcoal:Celite column chromatography (Eylar and

Jeanloz, 1962). The column was eluted successively with 40 ml of H_2O, 25 ml of 4% ethanol, 225 ml of 7.5% ethanol, 450 ml of 12% ethanol, and 200 ml of 13.5% ethanol. Fractions (5 ml each) 1-25 (yield, 3.0 mg) contained (upon rechromatography) Bands 5, 6, and 7 with a trace of Band 4. Fraction 26-48 (yield, 22.8 mg) contained Band 4 with only traces of the contaminants. Fraction 49-57 (yield, 38.7 mg) consisted exclusively of Band 4. The final fraction 57-188 (yield, 67.0 mg) consisted predominantly of Band 4, with traces of Bands 2 and 3. Fraction 49-57, used for all subsequent analyses, was found to be homogeneous by chromatography in three solvents (A, B, and C), and by high voltage paper electrophoresis at pH 9.2, 0.1 M borate buffer.

Composition. Examination by paper chromatography following hydrolysis in 1 N HCl (100°, 4 hrs) revealed roughly equimolar quantities of fucose, galactose, and galactosaminitol. No other sugars were detected. Hydrolysis in 0.05 N HCl (100°, 85 min) followed by paper chromatography gave a large amount of fucose with only traces of galactose and galactosaminitol, suggesting that fucose occupies a terminal position. The intact material gave 60.5% hexose by the anthrone reaction, 30.9% fucose (Dische and Shettles, 1948), and 28% galactose (galactose oxidase). The theoretical values are 58.2%, 27.6% and 30.7%, respectively.

Periodate oxidation. This was performed as previously described by Eylar and Jeanloz (1962a). Analysis for galactose of aliquots taken at timed intervals indicated that over 95% of this sugar was destroyed within 1 hour in both the sample and a standard of 2'fucosyllactose. This result strongly implied that positions 3 and 4 of the galactose are free.

Smith degradation. Approximately 10 mg of the purified material was oxidized with periodate for 10 hrs. Excess sodium arsenite was added to the reaction mixture, which was then reduced with excess $NaBH_4$, neutralized with acetic acid, deionized with Amberlite MB-3, and lyophilized. The residue was hydrolyzed in 0.1 N HCl at 80° for 1 hr., reduced again with $NaBH_4$, deionized and lyophilized. The residue was hydrolyzed in 1 N HCl at 100° for 4 hrs.,

evaporated to dryness, and chromatographed on paper with Solvent A. Three well-separated components (R_f's = 0.59, 0.48, and 0.27) were present. The two faster components co-chromatographed with 1,2-dihydroxypropane and glycerol, respectively. The presence of 1,2-dihydroxypropane, derived from fucose, eliminated the possibility of the latter existing in the furanose configuration; the absence of both arabitol and threitol (R_f's = 0.33 and 0.38, respectively) eliminates the possibility of a furanose configuration for galactose. The presence of glycerol, derived from galactose, confirms the above finding that the 3 and 4 positions of galactose are unsubstituted.

The third component (R_f 0.27) is derived from N-acetylgalactosaminitol. The three possible products, 2-amino-2-deoxyglycerol, 2-amino-2-deoxythreitol, and 2-amino-2-deoxyarabitol, form, together with 2-amino-2-deoxygalactitol, a homologous series of compounds, which, when plotted by the method described by French and Wild (1953) yield a linear relationship between chromatographic mobility and the number of carbon atoms. Using the data of Foster et al (1960) and the chromatographic mobility of 2-amino-2-deoxygalactitol (galactosaminitol) and serinol in Solvent B, the product was calculated to be the 5 carbon member of the series, 2-amino-2-deoxyarabitol (arabinosaminitol). These data demonstrate that galactose is joined to position 4 of the N-acetylgalactosaminitol. This conclusion agrees with the results obtained by exhaustive periodate oxidation of the native PSM, in which 70% of the galactosamine is found to be periodate-resistant. Thus, solely from the results of the periodate oxidation and the Smith degradation we can assign, with a fair degree of certainty, the following structure:

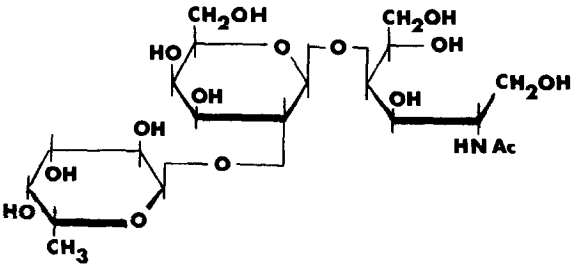
fucopyranosyl-(1→ 2 or 1→ 6)-galactopyranosyl-(1→ 4)-N-acetylgalactosaminitol.

Galactose Oxidase. In order to differentiate between the two possible linkages to galactose, galactose oxidase (Galactostat Kit, Worthington) was reacted with the purified material and 2'fucosyllactose. Positive reactions (12% and 13%, respectively, of theoretical) were obtained. An additional standard, 3'sialyllactose (General Biochemicals), also gave a reaction (0.6%

of theoretical). These data provide strong evidence for the linkage of the fucose to the galactose at position 2, rather than 6. In addition they rule out the possibility of a branched structure (in which fucose and galactose are both linked to the N-acetylgalactosaminitol), for in this case the galactose should react quantitatively (Avigad et al, 1962).

Methylation. Approximately 5 mg each of the purified material and 2'fucosyllactose as standard were subjected to Haworth methylation at 4°. The reaction mixtures were neutralized, deionized with Amberlite MB-3, and lyophilized. The residues were dried in vacuo over P_2O_5 and further methylated (Purdie technique) in dimethylformamide as described by Montgomery et al (1965). The reaction mixtures were again neutralized, deionized and lyophilized, and following hydrolysis in 1 N HCl at 100° for 2.5 hours, examined by paper chromatography with Solvents A, D, E, and F. With each solvent a component was derived from both starting materials which co-chromatographed with authentic 3,4,6-tri-O-methylgalactose. The 2,3,4- and 2,4,6-tri-O-methylgalactose derivatives moved slightly ahead of the 3,4,6- derivative in all 4 solvents. Only traces of galactose and fucose were observed, indicating nearly complete reaction in both cases. These data establish conclusively the linkage of fucose to galactose at position 2.

Conclusions. These results lead us to the conclusion that the trisaccharide derived from porcine submaxillary glycoprotein has the structure:



α-L-fucopyranosyl-(1→ 2)-β-D-galactopyranosyl-(1→ 4)-N-acetyl-D-galactosaminitol.

This trisaccharide, isolated from intact porcine submaxillary glycoprotein was also obtained from GP I and GP II. Work is currently in progress to determine if the trisaccharide is joined directly (by glycosidic linkage) to the serine or threonine residues of the protein (Katzman and Eylar, 1965), or if it possibly originates from a large oligosaccharide unit. It is of interest that several oligosaccharides isolated from human milk have been shown to contain a fucose unit joined to the 2 position of galactose (Kuhn et al, 1956).

Very recently Lloyd et al reported the isolation of tetra- and pentasaccharides from the blood group A, B, and H substances in which a terminal fucose residue is joined to the 2 position of galactose. It would appear, therefore, that the fucosyl-(1→ 2)-galactose linkage is a very general one that should be expected to occur in a wide variety of glycoproteins.

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