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Structure Determination of Oligosaccharides Isolated from A^+, H^+ and A^-H^- Hog-Submaxillary-Gland Mucin Glycoproteins, by 360-MHz ^{1}H-NMR Spectroscopy, Permethylation Analysis and Mass Spectrometry

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Alkaline borohydride reductive cleavage (β-elimination) of hog submaxillary glycoproteins from three immunologically determined phenotypes, viz. A^+, H^+ and A^-H^-, resulted in the release of a series of neutral and acidic oligosaccharide-alditols. 360-MHz ^{1}H-NMR spectroscopy in combination with methylation analysis and mass spectrometry were used for reinvestigation of the structures of these oligosaccharide-alditols. All are partial structures representing the possible complete and biosynthetically incomplete stages of the chain of a pentasaccharide-*N*-acetylgalactosaminitol, present in the glycoprotein with blood-group-A activity:

$$\begin{array}{lll} \text{GalNAc}(\alpha 1\rightarrow 3)\text{Gal}(\beta 1\rightarrow 3)\text{GalNAc-ol} & & \\ \quad\widehat{2} & \quad\widehat{6} & \\ \quad\uparrow & \quad\uparrow & \\ \quad 1 & \quad 2 & \\ \quad\underset{\sim}{\alpha} & \quad\underset{\sim}{\alpha} & \\ \quad\text{Fuc} & \quad\text{NeuGc} & \end{array}$$

In this way, a prolonged argument about the occurrence of a NeuGc($\alpha 2\rightarrow 6$)Gal moiety in these carbohydrate chains, suggested by Aminoff et al. [Aminoff, D., Baig, M. M. and Gathmann, W. D. (1979) *J. Biol. Chem. 254*, 1788–1793 and 8909–8913] has been brought to a definite end. In the investigated oligosaccharide-alditols *N*-glycoloylneuraminic acid (NeuGc) is in no case attached to galactose (Gal), but, if present, it is ($\alpha 2\rightarrow 6$)-linked to *N*-acetylgalactosaminitol (GalNAc-ol).

Three distinct phenotypes can be discerned immunologically in the aqueous extracts of mucins derived from individual hog submaxillary glands, namely: A^+, H^+, and A^-H^-; this was ascribed, at least in part, to differences in the oligosaccharide structures of the mucin glycoproteins [1]. Alkaline borohydride reductive cleavage (β-elimination reaction) is a useful method to release the oligosaccharides from their linkage to serine or threonine in the polypeptide backbone [2]. The availability of such a facile procedure for the cleavage of the carbohydrate chains from the protein core makes it possible to isolate the oligosaccharides, determine their structures, and thereby correlate structure with biological activity.

The first such complete accounting of the oligosaccharides of porcine submaxillary mucin was made by Carlson [3, 4] using A^+ or A^- porcine submaxillary glands. A subsequent study was undertaken by Aminoff and colleagues [5–7] in order to establish the role of the carbohydrate structure in determining the serological differences between A^+, H^+ and A^-H^- phenotypically active glycoproteins. In the studies of Carlson [3, 4] the structures of the isolated oligosaccharides were established by several techniques including periodate oxidation, enzymic methods and methylation analysis. The structures proposed by Aminoff et al. [5–7] were mainly based on periodate oxidation. The results of both investigations were controversial, as the results of Aminoff implicated the possible attachment of sialic acid in an ($\alpha 2\rightarrow 6$) linkage to the galactose, as well as to the protein-linked *N*-acetylgalactosamine residues of the oligosaccharide chains in porcine submaxillary mucin, whereas only the latter type occurs according to Carlson.

A thorough study, utilizing at least two independent methods for structural analysis, seemed to be required for bringing this controversy to an end. Since 360-MHz ^{1}H-NMR spectroscopy has proved to be a very powerful technique in the determination of carbohydrate structures [8, 9], this method in conjunction with methylation analysis and mass spectrometry was applied to shed light on this problem. A preliminary report on these findings has previously been presented [10].

MATERIALS AND METHODS

Details of the source, serological typing and isolation of the glycoproteins from hog submaxillary glands have been published [5–7].

The analytical methods and chromatographic procedures used in the isolation and characterization of the reduced oligosaccharides were also previously described [5–7].

Abbreviations. GalNAc, *N*-acetylgalactosamine; GalNAc-ol, *N*-acetylgalactosaminitol; Fuc, fucose; NeuGc, *N*-glycoloylneuraminic acid; NeuAc, *N*-acetylneuraminic acid; NMR, nuclear magnetic resonance.

360-MHz ^{1}H-NMR Spectroscopy

For ^{1}H-NMR spectroscopic analysis the neutralized oligosaccharide-alditols were repeatedly treated with ^2H_2O at room temperature. After each treatment with ^2H_2O the materials were lyophilized.

The 360-MHz ^{1}H-NMR spectra were recorded on a Bruker HX-360 spectrometer, operating in the Fourier transform mode at probe temperatures of 25 °C or 60 °C. Chemical shifts are given at 25 °C, relative to sodium 2,2-dimethyl-2-silapentane-5-sulphonate (indirectly to acetone in ^2H_2O: $\delta = 2.225$ ppm), with an accuracy of 0.003 ppm.

Preparation of Permethylated Oligosaccharide-Alditols

The oligosaccharide-alditols (0.5 µmol per sugar residue) were methylated according to Hakomori [11] as modified by Björndal et al. [12]. After extraction with chloroform, the methylated products were purified by passing through a silica gel column (0.5 × 5 cm, kieselgel highest purity, 70 – 325 mesh, Merck, Darmstadt, FRG). The column was washed with 5 ml chloroform and eluted with 2 ml methanol/chloroform (5 : 95, v/v) [13].

Gas-Liquid Chromatography and Mass-Spectrometry of the Permethylated Oligosaccharide-alditols

The permethylated neutral di-, tri-, and tetrasaccharide-alditols as well as the acidic di- and trisaccharide-alditols were analyzed by gas-liquid chromatography/mass spectrometry. Chromatography was done using a Girdel model 30 gas chromatograph (Suresnes, France); the glass column (0.3 × 100 cm) was packed with Supelcoport (100 – 120 mesh) containing 1 % Dexsil-300; column temperature: 150 – 320 °C with a temperature gradient of 4 °C/min; flow rate of carrier gas (helium): 20 ml/min. Mass spectrometry was carried out on a Riber-Mag 10 – 10 mass spectrometer (Rueil-Malmaison, France); electron energy: 70 eV; ionization current: 0.2 mA; chamber temperature: 190 °C. The retention times of the permethylated oligosaccharide-alditols were calculated relative to the permethylated maltotriose-alditol. The permethylated acidic tetra- and pentasaccharide-alditols were analyzed by direct-inlet mass spectrometry on this Riber-Mag 10-10 mass spectrometer; electron energy: 70 eV; ionization current: 0.2 mA; chamber temperature: 190 °C; probe temperature: 300 °C.

Identification of Partially Methylated Monosaccharides

The partially methylated monosaccharides obtained by methanolysis of permethylated oligosaccharide-alditols (0.5 M methanol/HCl for 24 h at 80 °C) were analyzed by gas-liquid chromatography after acetylation in pyridine/acetic anhydride (1 : 1, v/v, 500 µl, 100 °C, 30 min) using a Varian-Aerograph 1200 gas chromatograph (Orsay, France), equipped with a glass column (0.3 × 300 cm) packed with Chromosorb-W-HP (100 – 120 mesh) containing 3 % Carbowax 6000; column temperature: 125 – 200 °C with a temperature gradient of 2 °C/min; flow rate of carrier gas (nitrogen): 20 ml/min; and glass column (0.3 × 300 cm) packed with Chromosorb-W-HP (100 – 120 mesh) containing 3 % Silicone OV 17; column temperature: 140 – 300 °C with a temperature gradient of 6 °C/min; flow rate of carrier gas (nitrogen): 20 ml/min.

RESULTS

Isolation of acidic and neutral oligosaccharide-alditols was made in the same manner from each of the three phenotypes [5 – 7].

Composition of the Oligosaccharide-alditols

The composition and molar ratios of carbohydrates present in the purified oligosaccharide-alditols are given in Table 1.

Permethylation Studies

The molar ratios of the various partially methylated monosaccharides obtained on methanolysis and acetylation of the permethylated oligosaccharide-alditols are given in Table 2.

From the substitution pattern of the GalNAc-ols it can be concluded that these residues in the neutral oligosaccharide-alditols and the acidic disaccharide-alditols are glycosidically linked via C-3 and C-6, respectively. In the acidic oligosaccharide-alditols with more than two sugar residues, both C-3 and C-6 of the GalNAc-ol are involved in linkages.

In the neutral trisaccharide-alditol, Gal(1→3)GalNAc-ol is substituted by Fuc in position C-2 of Gal. The neutral tetrasaccharide-alditol can be conceived as an extension of the trisaccharide-alditol containing an additional GalNAc, linked (1→3) to Gal.

The acidic oligosaccharide-alditols contain NeuGc, linked to C-6 of GalNAc-ol, provided that the acidic compounds are considered to be built up from the neutral ones by extension with NeuGc. The structures proposed on the basis of methylation analysis were verified by gas chromatographic and mass spectrometric analysis of the permethylated intact oligosaccharides. Gas-liquid chromatography on a Dexsil column gives a good separation of the permethylated neutral mono-, di-, tri- and tetra-, as well as of the acidic di- and trisaccharide-alditols. The retention times of these compounds are listed in Table 3. The permethylated acidic tetra- and pentasaccharide-alditols are not sufficiently volatile for analysis by gas-liquid chromatography.

The mass spectra and fragmentation patterns of seven permethylated oligosaccharide-alditols are shown in Fig. 1. The nomenclature of the fragmentations is described elsewhere [22 – 24]. In the mass spectrum of the permethylated reduced neutral disaccharide (Fig. 1 A) the aA fragment ions at *m/e* 219 (aA_1), *m/e* 187 (aA_2) and *m/e* 155 (aA_3) show the occurrence of a terminal non-reducing hexose, whereas for the alditol the fragment ion at *m/e* 276 (ald) is characteristic. The (1→3) linkage can be derived from the fragment ions at *m/e* 133 (101), 378 and 422.

The occurrence of a non-reducing 6-deoxyhexose in the reduced neutral trisaccharide is evident from the aA fragment ions at *m/e* 189 (aA_1), *m/e* 157 (aA_2) (Fig. 1 B). The series of baA fragment ions at *m/e* 393 (baA_1), *m/e* 361 (baA_2) and *m/e* 329 (baA_3) indicates a 6-deoxyhexose → hexose sequence, while a hexose → hexosaminitol sequence can be derived from the b-ald fragment ions at *m/e* 480 (b-ald_1), *m/e* 448 (b-ald_2) and *m/e* 416 (b-ald_3). Since the sum of the intensities of the baA fragment ions is larger than the sum of the b-ald fragment ions, the type of linkage between the deoxyhexose and the hexose is (1→2) rather than (1→4) or (1→6) [14]. Further, the fragment ions at *m/e* 133 (101), *m/e* 552 and *m/e* 596 show that the alditol is glycosylated at C-3.

In the mass spectrum of the permethylated reduced neutral tetrasaccharide (Fig. 1 C) the fragment ions aA (at *m/e* 189 and

Table 1. *The composition and molar ratios of carbohydrates in the oligosaccharide-alditols*
The molar sugar composition of the neutral oligosaccharide-alditols was calculated on the basis of one residue of GalNAc-ol, and that of the acidic oligosaccharide-alditols on the basis of one residue of NeuGc

Oligosaccharide-alditol	Obtained from phenotype	Monosaccharide residues				
		GalNAc-ol	Gal	Fuc	NeuGc	GalNAc
		mol/mol oligosaccharide				
Neutral monosaccharide		1.00	0	0	0	0
Neutral disaccharide		1.00	0.95	0	0	0
Neutral trisaccharide		1.00	0.94	1.06	0	0
Neutral tetrasaccharide		1.00	1.04	0.98	0	0.96
Acidic disaccharide	A^+	0.97	0	0	1.00	0
	H^+	1.03	0	0	1.00	0
	A^-H^-	0.96	0	0	1.00	0
Acidic trisaccharide	A^+	1.02	1.03	0	1.00	0
	H^+	0.88	0.93	0	1.00	0
	A^-H^-	0.97	0.98	0	1.00	0
Acidic tetrasaccharide	A^+	0.96	1.00	0.95	1.00	0
	H^+	0.96	1.07	0.99	1.00	0
	A^-H^-	1.03	1.07	0.99	1.00	0
Acidic pentasaccharide	A^+	1.04	1.07	1.02	1.00	0.96

Table 2. *Molar ratios of monosaccharide methyl ethers present in the methanolysates of the permethylated reduced oligosaccharides derived from A^+, H^+ or A^-H^- hog submaxillary glycoproteins*
2,3,4-Me_3Fuc = 2,3,4-tri-*O*-methyl-fucose, etc.; GalNAcNMe = *N*-acetyl-*N*-methyl-galactosamine etc.

Oligosaccharide-alditol	Obtained from phenotype	Partially methylated monosaccharides								
		2,3,4-Me_3Fuc	2,3,4,6-Me_4Gal	3,4,6-Me_3Gal	4,6-Me_2Gal	3,4,6-Me_3Gal-NAcNMe	4,7,8,9-Me_4Neu-GcNMe	1,4,5,6-Me_4Gal-NAcNMe-ol	1,3,4,5-Me_4Gal-NAcNMe-ol	1,4,5-Me_3Gal-NAcNMe-ol
		mol/mol oligosaccharide								
Neutral disaccharide		0	1.0	0	0	0	0	0.87	0	0
Neutral trisaccharide		0.91	0	1.0	0	0	0	0.85	0	0
Neutral tetrasaccharide		0.89	0	0	1.0	0.80	0	0.90	0	0
Acidic disaccharide	A^+	0	0	0	0	0	1.0	0	0.84	0
	H^+	0	0	0	0	0	1.0	0	0.89	0
	A^-H^-	0	0	0	0	0	1.0	0	0.87	0
Acidic trisaccharide	A^+	0	1.0	0	0	0	0.95	0	0	0.79
	H^+	0	1.0	0	0	0	0.98	0	0	0.80
	A^-H^-	0	1.0	0	0	0	0.92	0	0	0.86
Acidic tetrasaccharide	A^+	0.94	0	1.0	0	0	0.97	0	0	0.70
	H^+	0.88	0	1.0	0	0	0.94	0	0	0.84
	A^-H^-	0.91	0	1.0	0	0	0.96	0	0	0.87
Acidic pentasaccharide	A^+	0.92	0	0	1.0	0.81	0.94	0	0	0.80

157) and a'A (at *m/e* 260 and 228) indicate the occurrence of both a 6-deoxyhexose and a hexosamine in terminal non-reducing position. The fragment ion at *m/e* 276 is characteristic for the mono-substituted hexosaminitol. The presence of the baa' fragment ion at *m/e* 638 demonstrates that the 6-deoxy-hexose and hexosamine are both linked to the same hexose unit. The fragment ion at *m/e* 133 (101) proves the linkage between hexose and hexosaminitol to be (1→3).

The gas-liquid chromatographic and mass-spectrometric data of corresponding acidic oligosaccharide-alditols obtained from glycoproteins with A^+, H^+ or A^-H^- immunological activity appeared to be identical. In all acidic oligosaccharide-alditols the presence of terminal NeuGc is evident from the fragment ions at *m/e* 406 and 374 in the mass spectra of their permethylated derivatives (Fig. 1 D–G). From the mass spectrum of the permethylated acidic disaccharide-alditol (Fig. 1 D)

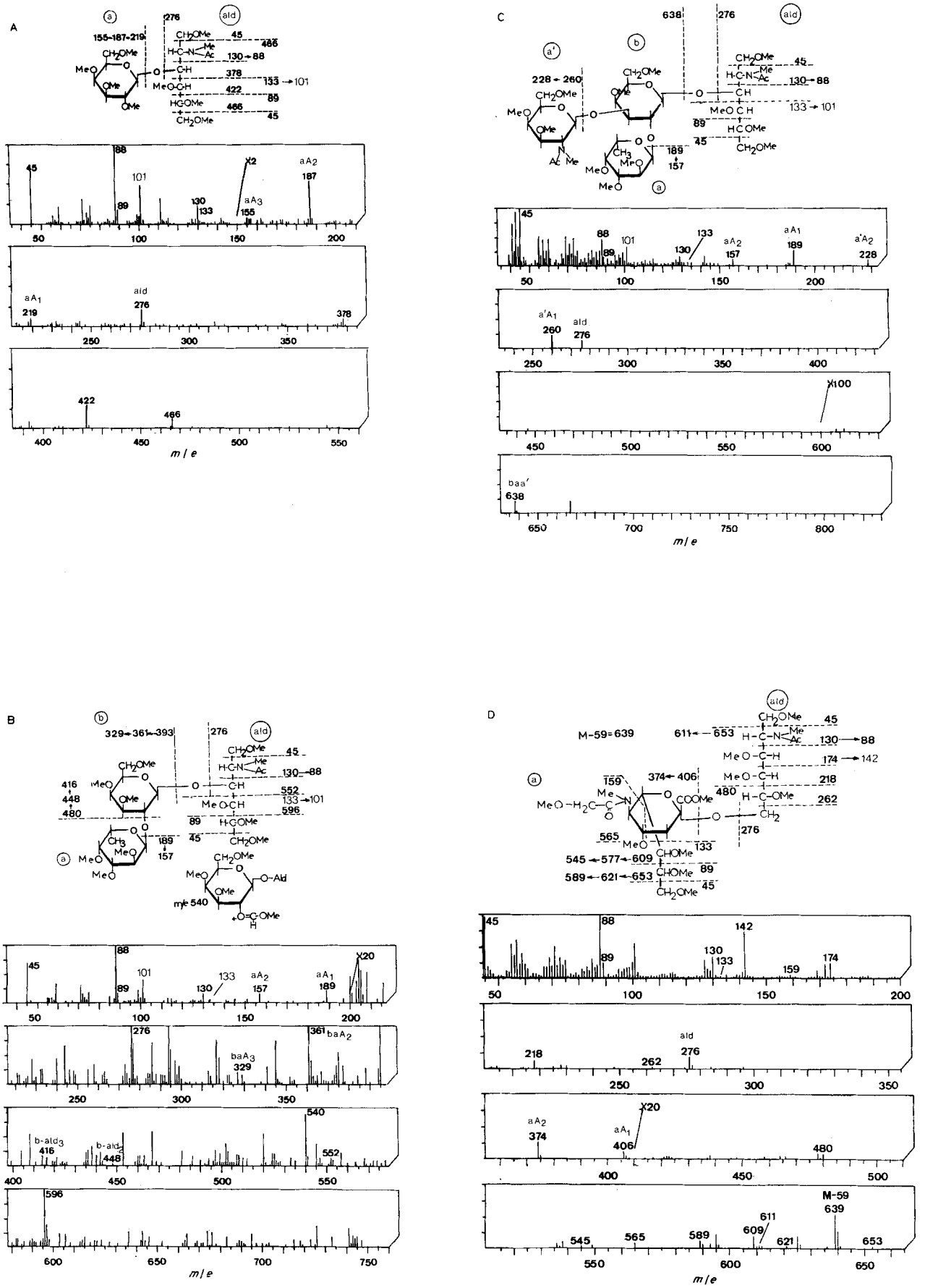
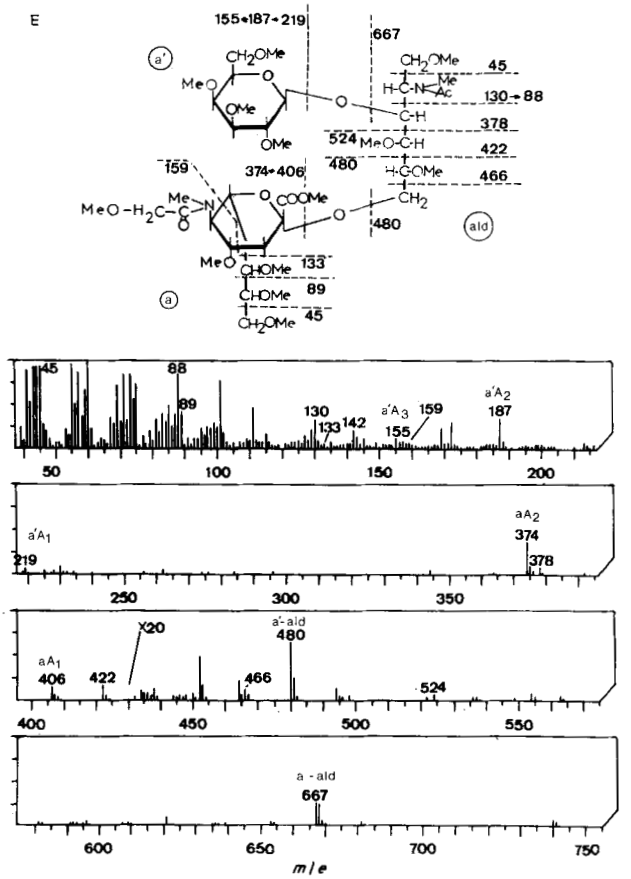
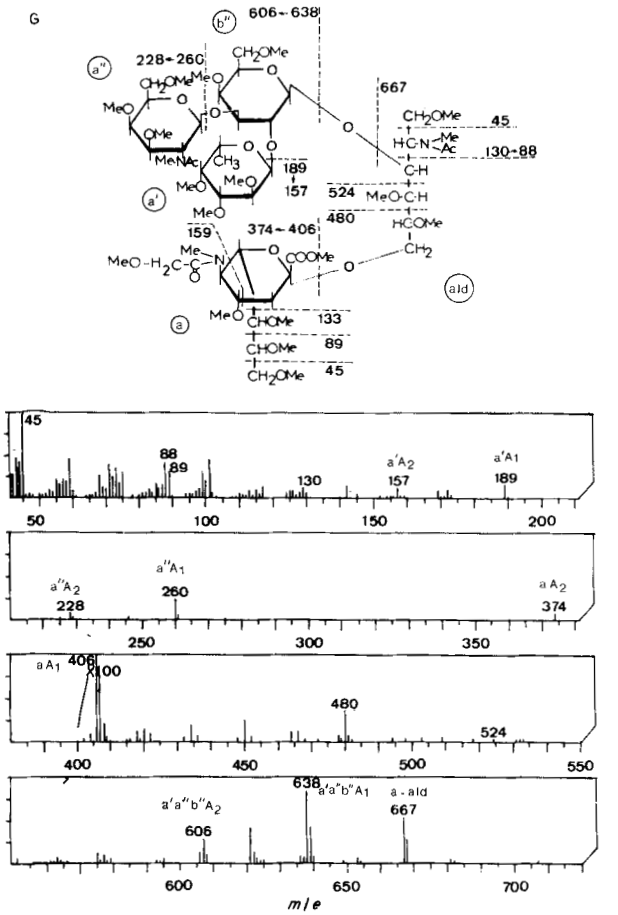


Fig. 1. *Mass spectra and fragmentation patterns of permethylated oligosaccharide-alditols obtained after alkaline borohydride treatment of A^+, H^+ and A^-H^- active hog submaxillary glycoproteins, and subsequent permethylation*





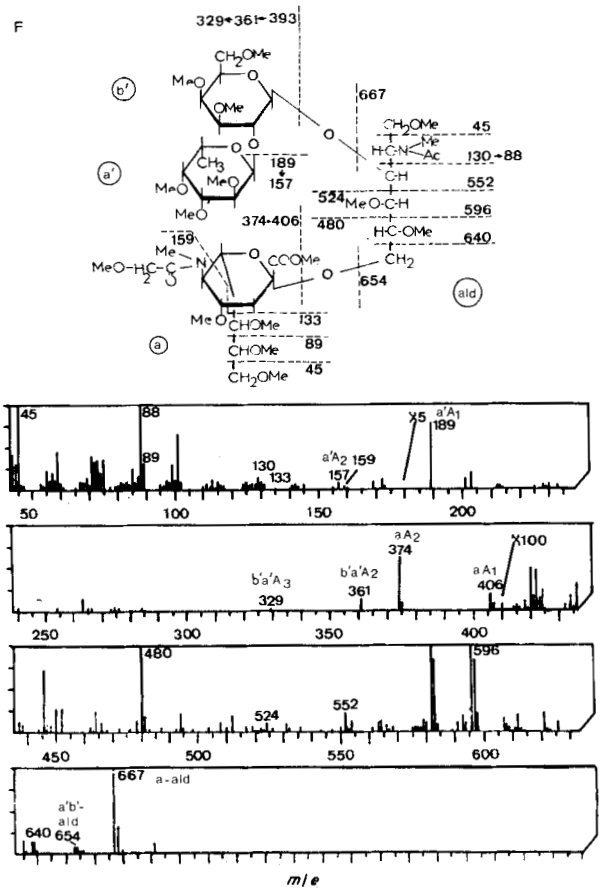


Table 3. *Retention times of permethylated oligosaccharide-alditols relative to permethyl maltotriose-alditol on 1% Dexsil-300*
The absolute retention time of permethyl maltotriose-alditol is 6.7 min

Permethylated reduced oligosaccharide	Relative retention time
Neutral monosaccharide	0.19
Neutral disaccharide	0.53
Neutral trisaccharide	1.51
Acidic disaccharide from A^+, H^+ or A^-H^-	1.95
Acidic trisaccharide from A^+, H^+ or A^-H^-	2.84
Neutral tetrasaccharide	3.13

it can be concluded that this NeuGc residue is linked to C-6 (*m/e* 262 and 480) of a hexosaminitol (*m/e* 276). The occurrence of a non-reducing hexose in the acidic trisaccharide-alditol is evident from the a′A fragment ions at *m/e* 219 ($a'A_1$), *m/e* 187 ($a'A_2$) and *m/e* 155 ($a'A_3$) (Fig. 1E). A (1→3) linkage between hexose and hexosaminitol can be derived from the fragment ion *m/e* 378, while the ion at *m/e* 480 indicates a (2→6) linkage of NeuGc to the hexosaminitol. The acidic tetrasaccharide-alditol (see Fig. 1F) is also a branched reduced oligosaccharide since the fragment ion at *m/e* 276 (ald) is absent. The NeuGc residue [terminal position: *m/e* 406 (aA_1) and *m/e* 374 (aA_2)] is attached to C-6 of the *N*-acetylhexosaminitol (*m/e* 480). A 6-deoxyhexose → hexose → hexosaminitol sequence can be deduced from the a′A fragment ions at *m/e* 189 ($a'A_1$) and *m/e* 157

Table 4. ^1H *Chemical shifts of characteristic protons of constituent monosaccharides for the reduced oligosaccharides from* A^+, H^+ *and* A^-H^- *hog*

Compound	Obtained from phenotype	Chemical shift of GalNAc-ol protons								
		H-1	H-1′	H-2	H-3	H-4	H-5	H-6	H-6′	NAc
		ppm								
Neutral monosaccharide		3.74	3.67	4.255	3.847	3.385	3.928	3.67	3.64	2.055
Neutral disaccharide		3.81	3.73	4.396	4.062	3.506	4.198	3.69	3.65	2.050
Neutral trisaccharide		3.80	3.7–3.8	4.398	4.089	3.520	4.162	3.68	3.63	2.046
Neutral tetrasaccharide		3.7–3.8	3.7–3.8	4.302	4.100	3.606	4.125	3.68	3.64	2.042[a]
Acidic disaccharide	A^+	3.73	3.66	4.253	3.842	3.416	4.025	3.842	3.536	2.057
	H^+			4.251		3.412	4.029			2.057
	A^-H^-			4.253		3.415	4.031			2.057
Acidic trisaccharide	A^+	3.7–3.8	3.7–3.8	4.383	4.061	3.534	4.251	3.860	3.491	2.049
	H^+			4.383	4.063	3.534	4.244		3.490	2.047
	A^-H^-			4.383	4.063	3.534	4.251		3.491	2.049
Acidic tetrasaccharide	A^+	3.86	3.75	4.386	4.087	3.545	4.226	3.84	3.484	2.043
	H^+			4.384	4.081		4.223		3.482	2.043
	A^-H^-			4.385	4.085		4.226		3.483	2.043
Acidic pentasaccharide	A^+	3.7–3.8	3.7–3.8	4.291	4.089	3.624	4.185	3.862	3.493	2.044[b]

a,b Assignments may be interchanged

($a'A_2$) and the $b'a'A$ ions at *m/e* 393, 361 and 329. The fragment ion at *m/e* 552 is indicative of substitution at C-3 of the hexosaminitol. In the acidic pentasaccharide-alditol (Fig. 1G) the terminal NeuGc is linked again (2→6) to the hexosaminitol (*m/e* 480). The a′A (at *m/e* 189 and 157) and a″A fragment ions (at *m/e* 260 and 228) show the occurrence of both a 6-deoxyhexose and a hexosamine in terminal non-reducing position. The latter are linked to the same hexose unit (evident from *m/e* 638 and 606). The fragment ion at *m/e* 524 proves the linkage between hexose and hexosaminitol to be (1→3).

The samples of corresponding oligosaccharide-alditols, obtained from glycoproteins with A^+, H^+ or A^-H^- immunological activity, gave identical results.

The 360-MHz ^1H-NMR Studies

For the interpretation of the 360-MHz ^1H-NMR spectra of the oligosaccharide-alditols in terms of structural assignments, the resonances of the anomeric protons, the skeleton protons of GalNAc-ol, the H-3 protons of NeuGc residues, the H-5 and H-6 protons of Fuc residues, the H-2, H-3 and H-4 protons of Gal residues and the *N*-acetyl methyl and *N*-glycoloyl methylene protons were used. The chemical shifts of these protons in the various oligosaccharide-alditols are compiled in Table 4.

First, the assignment of all signals in GalNAc-ol, obtained from GalNAc by reduction with $NaBH_4$ or NaB^2H_4, was carried out. The chemical shifts and coupling constants of GalNAc-ol were refined by computer simulation of the spectra.

The spectrum of the neutral disaccharide-alditol shows, in comparison to that of GalNAc-ol, shift increments for all skeleton protons. The relatively large shift increment of H-5 ($\Delta\delta$ = 0.27 ppm) is remarkable, since the shifts of H-6 and H-6′ are almost unaffected. It may result from a 1,3-diaxial-like interaction with H-3. The linkage of Gal to C-3 of GalNAc-ol is evident from the relatively large shift increment of H-3 ($\Delta\delta$ = 0.22 ppm). The coupling constant $J_{1,2}$ (7.3 Hz) of Gal is indicative of a β-glycosidic linkage.

The chemical shifts of the GalNAc-ol protons in the neutral trisaccharide-alditol are almost identical to those of the reduced disaccharide. The presence of Fuc in (1→2) linkage to Gal gives rise to shift increments of H-1, H-2, H-3 and H-4 of Gal. The value of $J_{1,2}$ of Fuc (4.0 Hz) points to an α-glycosidic linkage.

In the neutral tetrasaccharide-alditol the attachment of GalNAc to C-3 of Gal is reflected by the relatively large shift increment of H-3 of Gal ($\Delta\delta$ = 0.37 ppm), in comparison to the trisaccharide-alditol. Fuc as well as GalNAc are α-glycosidically linked, as can be concluded from the coupling constants ($J_{1,2}$ = 4.0 Hz and $J_{1,2}$ = 2.0 Hz, respectively, cf. [15]). It has to be noted that the assignment of H-1 for Fuc and GalNAc was carried out on the basis of these coupling constants, since the chemical shift of H-1 of Fuc differs considerably from that in the trisaccharide-alditol. The assignment is in agreement with ^1H-NMR spectral data from milk oligosaccharides (unpublished results). From the foregoing the following structure of the tetrasaccharide-alditol can be inferred: GalNAc(α1→3)[Fuc(α1→2)]Gal(β1→3)GalNAc-ol. The NMR spectra of corresponding acidic oligosaccharide-alditols, obtained from A^+, H^+ or A^-H^- active hog submaxillary glycoproteins, turned out to be identical. For this reason a complete set of data is only given for each A^+ component in Table 4.

The acidic disaccharide-alditol contains a NeuGc residue (α2→6)-linked to GalNAc-ol. The set of chemical shift values for the H-3 protons of NeuGc ($\delta_{H\text{-}3ax}$ = 1.721 ppm; $\delta_{H\text{-}3eq}$ = 2.745 ppm) is indicative of an α-type of linkage [16]. For the attachment of NeuGc to C-6 of the alditol the following spectral features are characteristic [17]: (a) the H-6 and H-6′ of GalNAc-ol become less equivalent, as is expressed by the oppositely directed changes in their chemical shifts as compared to GalNAc-ol; (b) the geminal coupling constant $J_{6,6'}$, changes from −11.7 Hz in GalNAc-ol to −9.8 Hz in the acidic disaccharide-alditol.

Analogously, the higher acidic oligosaccharide-alditols differ from the corresponding neutral ones only in the attach-

Chemical shift of Gal protons				Chemical shift of Fuc protons			Chemical shift of GalNAc protons		Chemical shift of NeuGc protons		
H-1	H-2	H-3	H-4	H-1	H-5	H-6	H-1	NAc	H-3ax	H-3eq	NGc
ppm											
–	–	–	–	–	–	–	–	–	–	–	–
4.475	3.564	3.754	3.900	–	–	–	–	–	–	–	–
4.584	3.688	3.880	3.926	5.256	4.279	1.243	–	–	–	–	–
4.720	3.90	4.254	4.224	5.189	4.343	1.237	5.389	2.048[a]	–	–	–
–	–	–	–	–	–	–	–	–	1.721	2.746	4.124
–	–	–	–	–	–	–	–	–	1.721	2.745	4.125
–	–	–	–	–	–	–	–	–	1.721	2.742	4.122
4.477	3.574	3.669	3.899	–	–	–	–	–	1.714	2.743	4.122
4.475				–	–	–	–	–	1.711	2.747	4.120
4.477				–	–	–	–	–	1.713	2.744	4.124
4.586	3.697	3.870	3.915	5.271	4.271	1.239	–	–	1.714	2.747	4.122
4.586				5.267	4.271	1.236	–	–	1.712	2.743	4.120
4.588				5.271	4.275	1.237	–	–	1.715	2.745	4.122
4.719	3.908	4.248	4.023	5.184	4.335	1.242	5.395	2.046[b]	1.711	2.750	4.123

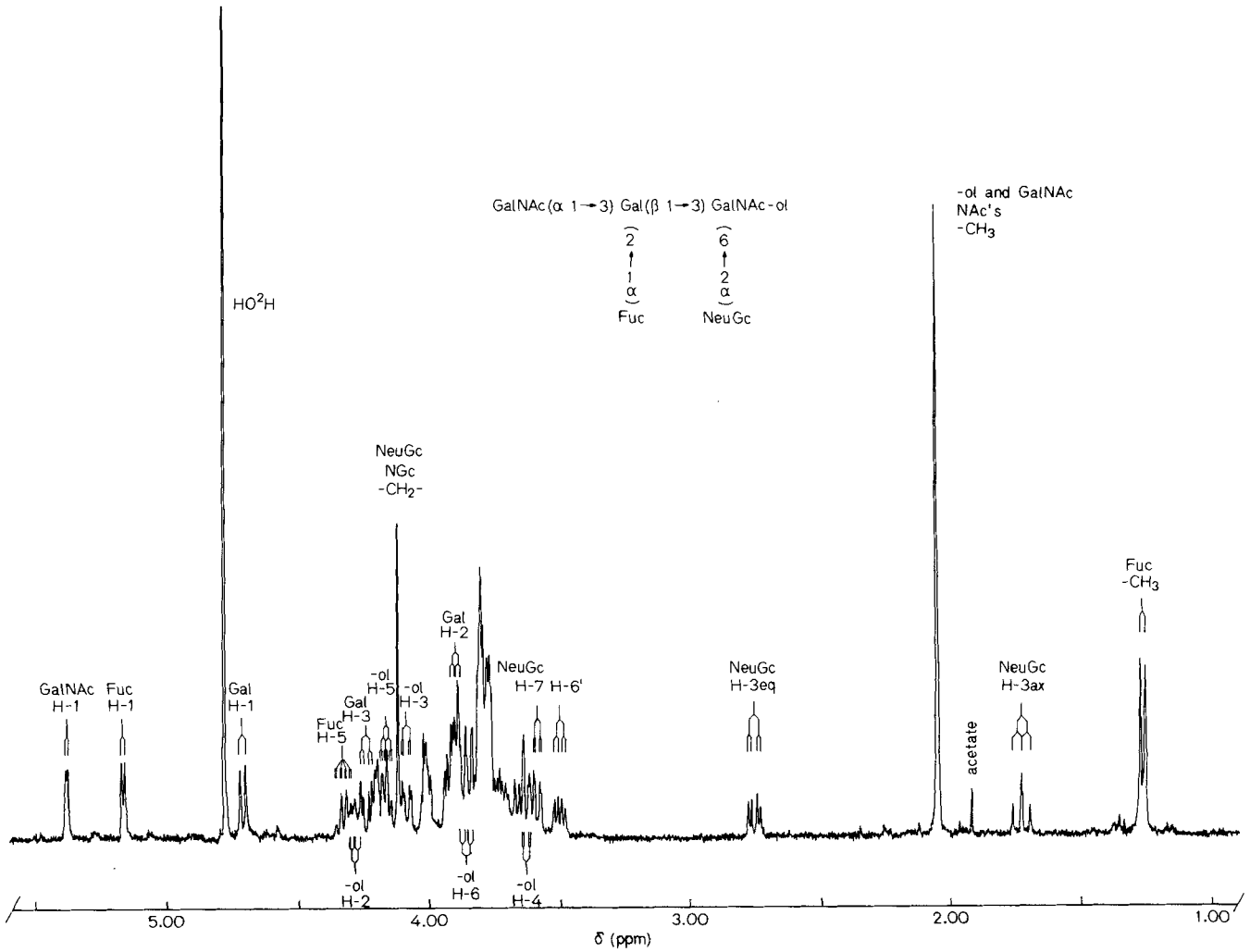


Fig. 2. *360-MHz ^{1}H-NMR spectrum of the acidic pentasaccharide-alditol in ^2H_2O at 25 °C*

ment of sialic acid, linked (α2→6) to GalNAc-ol. Comparison of the spectra of the acidic trisaccharide-alditol and the neutral disaccharide-alditol shows that the attachment of NeuGc affects considerably only the chemical shifts of H-6 and H-6′ of GalNAc-ol. In combination with the value of $J_{6,6'}$ (−9.8 Hz), the shift increment for H-6 and the shift decrement for H-6′ are indicative of linkage to C-6 of the hexosaminitol. The set of the H-3 chemical shifts of NeuGc ($\delta_{H\text{-}3ax}$ = 1.713 ppm; $\delta_{H\text{-}3eq}$ = 2.744 ppm) again points to α-linkage. It has to be stressed here that NeuGc is not linked to Gal in any position, since $\delta_{H\text{-}1}$ of Gal in the acidic trisaccharide-alditol is 4.477 ppm (not altered by the presence of NeuGc) [18, 19].

The acidic tetrasaccharide-alditol can be conceived as the neutral trisaccharide-alditol extended with a NeuGc moiety. The same relation exists between the acidic pentasaccharide-alditol and the neutral tetrasaccharide-alditol. The 360-MHz ^{1}H-NMR spectrum of the acidic pentasaccharide-alditol is given in Fig. 2.

Extension of the structures from the acidic di- to the pentasaccharide-alditol by successive attachment of Gal, Fuc and finally GalNAc can be deduced from the respective spectra in the same way as described for the series of neutral oligosaccharide-alditols.

An interesting feature in the spectra of the sialo compounds is the position of the H-3 axial and equatorial signals of NeuGc. The chemical shifts of these protons are known to be very sensitive to the sequentional arrangement of the sialic acid residue [18, 20]. The set of chemical shifts, δ = 1.721 ppm and δ = 2.744 ppm, can be considered as unique for a NeuGc(α2→6)GalNAc-ol sequence. Attachment of Gal (β1→3)-linked to GalNAc-ol causes a small but significant shift decrement of H-3 axial from 1.721 ppm to 1.713 ppm.

DISCUSSION

Structural studies by Aminoff et al. [5–7] on the oligosaccharides of porcine submaxillary mucin glycoproteins employing periodate oxidation indicated that sialic acid was linked to galactose as well as to *N*-acetylgalactosamine in A^+, H^+ and A^-H^- glycoproteins.

Examination of the same oligosaccharides by 360-MHz ^{1}H-NMR spectroscopy and methylation analysis of the neutral and acidic oligosaccharide-alditols, as well as by mass spectrometry of the permethylated intact oligosaccharide-alditols, prove that these compounds have the structures shown in Fig. 3. None of these structures contains the NeuGc(α2→6)Gal moiety. The following remarks are appropriate. (a) A hierarchy of complexity, from the simplest monosaccharide (GalNAc) to the most complex pentasaccharide is observed; it is interesting

neutral oligosaccharide-alditols		*acidic oligosaccharide-alditols*		obtained from phenotype
			NeuGc α2↓6 GalNAc-ol	
monosaccharide	GalNAc-ol	*disaccharide*		A^+, H^+, A^-H^-
			NeuGc α2↓6 Gal(β1→3)GalNAc-ol	
disaccharide	Gal(β1→3)GalNAc-ol	*trisaccharide*		A^+, H^+, A^-H^-
			NeuGc α2↓6 Gal(β1→3)GalNAc-ol 2↑1α Fuc	
trisaccharide	Gal(β1→3)GalNAc-ol 2↑1α Fuc	*tetrasaccharide*		A^+, H^+, A^-H^-
			NeuGc α2↓6 GalNAc(α1→3)Gal(β1→3)GalNAc-ol 2↑1α Fuc	
tetrasaccharide	GalNAc(α1→3)Gal(β1→3)GalNAc-ol 2↑1α Fuc	*pentasaccharide*		A^+ only

Fig. 3. *Proposed structures for the neutral and acidic oligosaccharide-alditols isolated from A^+, H^+ and A^-H^- hog submaxillary glycoproteins*

to note that the structures fit the scheme of biosynthetic pathways for this type of oligosaccharides [21]. (b) Both H^+ and A^-H^- phenotypes contain oligosaccharides of identical structures consisting of three acidic and three neutral oligosaccharides. (c) The A^+ glycoprotein contains two additional oligosaccharides, a neutral tetrasaccharide and an acidic pentasaccharide, which are not found in H^+ and A^-H^-, due to the presence of an additional GalNAc residue, completing the blood-group-A determinant.

A discrepancy exists between the data obtained by NMR spectroscopy and methylation studies, as compared to structures previously derived from periodate oxidation data [7]. Concerning the periodate oxidation studies of the acidic tri- and tetrasaccharide from porcine submaxillary gland mucin no obvious explanation can be given for the erroneous structures proposed [7]. This study lends no support to oligosaccharide structures with sialic acid linked ($\alpha 2 \rightarrow 6$) to galactose [5 – 7]; it confirms the structures proposed by Carlson [4].

The occurrence of sialic acid linked ($\alpha 2 \rightarrow 6$) to *N*-acetylgalactosaminitol in all acidic oligosaccharide-alditols from porcine submaxillary mucin makes it necessary to look for another explanation for observed A^-H^- immunological properties. It could be that secondary structural differences play a role in the serological activity of glycoproteins [7].

With regard to the physical techniques applied for the characterization of the compounds, mass spectrometry was found to be of great value for the analysis of the permethylated intact oligosaccharide-alditols. For the underivatized compounds the non-destructive high-resolution ^{1}H-NMR spectroscopy proved to be an effective method in the unraveling of primary structures. This shows that high-resolution ^{1}H-NMR spectroscopy is as suitable for the identification of *O*-glycosidically linked carbohydrate chains as it is for the *N*-glycosidically linked moieties, derived from glycoproteins.

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