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STRUCTURAL ANALYSIS BY FAST-ATOM-BOMBARDMENT MASS SPECTROMETRY OF THE MIXTURE OF ALDITOLS DERIVED FROM THE *O*-LINKED OLIGOSACCHARIDES OF MURINE GLYCOPHORINS*

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ABSTRACT

The *O*-glycosylically linked oligosaccharides from glycophorins of BALB/c mouse erythrocytes were released as a mixture of alditol derivatives on reductive β-elimination. A new approach, based on periodate oxidation in combination with f.a.b.-m.s., was used to elucidate the structure of one of the branched derivatives in the mixture. Evidence for the anomeric configuration was obtained by 500-MHz ^{1}H-n.m.r. spectroscopy. The following structures were found:

α-Neu5Ac-(2→3)-β-Gal-(1→3)-GalNAc-ol

α-Neu5Ac-(2→6)
GalNAc-ol
αNeu5Ac-(2→3)-β-Gal-(1→3)

α-Neu5Ac-(2→3)-β-Gal-(1→4)-β-GlcNAc-(1→6)
GalNAc-ol
α-Neu5Ac-(2→3)-β-Gal-(1→3)

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INTRODUCTION

Glycophorins are sialoglycoproteins of the erythrocyte membranes which contain multiple *O*-glycosylic oligosaccharides and carry most of the cell-surface sialic acid. The glycophorins of human red-cell membranes have been studied most thoroughly and the structures of three of them have been elucidated completely (glycophorin A) or almost completely (glycophorin B and C)[1,2]. The carbohydrate moieties of human glycophorins consist mainly of an *O*-linked tetrasaccharide and a smaller proportion of a linear trisaccharide, which correspond to di- and mono-sialylated forms of β-Gal-(1→3)-GalNAc, respectively[3,4]. The polypeptide and carbohydrate moieties of the glycophorins of some animal erythrocytes have been characterised[5]. Studies of murine glycophorins are less advanced, despite the wide use of mice and rats as experimental animals. The chemical characteristics of murine erythrocytes reported so far include the presence of *N*-acetylneuraminic acid and its *O*-acetylated derivatives in proportions depending on the strain[6,7]. Furthermore, the presence of two glycophorins, one containing *O*-glycosylic chains and the other both *O*- and *N*-glycosylic chains, has been described[8,9]. We now describe the structural analysis of the alditol derivatives of the *O*-linked oligosaccharides of murine glycophorins obtained after reductive β-elimination, without separation of the single components, by the use of chemical analyses, ^{1}H-n.m.r. spectroscopy, and periodate oxidation in combination with f.a.b.-m.s.[10].

EXPERIMENTAL

Glycophorins. — (a) Preparation. Fresh blood from BALB/c mice was treated with heparin (20 U/mL), and the erythrocytes were washed three times with saline containing 0.02M phosphate buffer (pH 7.4) (PBS), with careful removal of the buffy coat of the lymphocytes after each washing. The membranes were isolated according to the procedure of Dodge *et al.*[11]. The sialoglycoproteins (glycophorins) were obtained from the membranes by phenol–water extraction[12].

(b) Degradation with sodium hydroxide/sodium borohydride. The conditions[4] of the Carlson degradation were used. Briefly, a 0.4% solution of sialoglycoproteins in 0.05M NaOH/M $NaBH_4$ was left for 18 h at 50°. In some experiments, NaB^3H_4 was added to the reaction mixture. The excess of borohydride was decomposed with aqueous 50% acetic acid and the products were eluted from a column of Sephadex G-15 with water.

Analytical methods. — Neutral sugars were determined by the phenol–sulfuric acid method[13] and sialic acid by the method of Jourdian *et al.*[14] in untreated samples and in samples *O*-deacetylated[6] by treatment with 0.1M NaOH for 45 min at 0°. Monosaccharides were determined by g.l.c. as alditol acetates[15] after hydrolysis in 4M trifluoroacetic acid for 4 h at 100°. Methylation analysis was carried out as previously described[16,17]. Oligosaccharide-alditols were desialylated by hydrolysis in 0.05M H_2SO_4 for 1 h at 80°, followed by neutralisation with AG3-X4A

(HO^-) resin (BioRad). Oligosaccharide-alditols were oxidised with periodate[18] in 0.1M acetate buffer (pH 5.5) containing 8mM sodium periodate, for 48 h at 4°. Excess of periodate was reduced with ethylene glycol (10 μL) and the pH was adjusted to 7.0 prior to reduction with $NaBD_4$. The products were analysed by f.a.b.-m.s. after acetylation, using the procedure primarily designed for methylated oligosaccharides[10]. For g.l.c., a Hewlett–Packard 5890 instrument was used, equipped with a flame-ionisation detector. Separations were performed on W.C.O.T. capillary columns DB-225 (alditol acetates) and SE-30 (partially methylated alditol acetates).

Spectroscopic methods. — G.l.c.–m.s. (electron impact) was carried out on a VG 12-250 quadrupole instrument fitted with an SE-30 W.C.O.T. capillary column. Spectra were recorded at 70 eV with an ion-source temperature of 200°. For f.a.b.-m.s. (positive ion mode), a VG ZAB SE instrument was used with thioglycerol (1-thio-2,3-propanediol) as the matrix and xenon with a kinetic energy of 8 keV. ^{1}H-N.m.r. spectra were recorded at 25° with a Bruker AM-500 F.t.-spectrometer. The oligosaccharides were exchanged with ^2H_2O, lyophilised, and dissolved in high purity ^2H_2O (99.95%). The chemical shifts were expressed relative to internal sodium 4,4-dimethyl-4-silapentane (DSS), but measured relative to internal acetone (2.225 p.p.m.).

RESULTS

Preparation of mouse glycophorins. — The sialoglycoprotein (glycophorin) fraction from BALB/c mouse erythrocyte membranes was obtained in a yield of 16–22 mg per 100 mL of fresh blood. It has been reported[8] that two different glycophorins are present in this fraction, which contain carbohydrate components characteristic of O-linked chains (GalNAc, Gal, sialic acid), and probably only one of these glycophorins contained N-linked chain(s) as revealed by the presence of D-mannose, L-fucose, and an increased GlcNAc:GalNAc ratio. Colorimetric determination[14] of the sialic acid showed a >2-fold increase after *O*-deacetylation of the sample. This finding accorded with the report by Reuter *et al.*[7] that a major or unique form of sialic acid on erythrocytes of BALB/c mice is 9-*O*-acetyl-*N*-acetylneuraminic acid, which gives ~40% of the colour in the assay compared to that for *N*-acetylneuraminic acid[19].

Isolation of oligosaccharide derivatives and glycopeptides. — The glycophorin fraction was submitted to a reductive β-elimination reaction and the products were fractionated on Sephadex G-15 (Fig. 1). Two carbohydrate-containing peaks were combined and re-chromatographed. Their carbohydrate composition (Table I) indicated that the major components of fractions 1 and 2 were derivatives of the *N*-glycosylic and *O*-glycosylic chains, respectively. The persistence of GlcNAc in fraction 2 suggested the existence of GlcNAc-containing O-linked oligosaccharide derivatives.

Structural analysis of the derivatives in fraction 2. — The oligosaccharide-

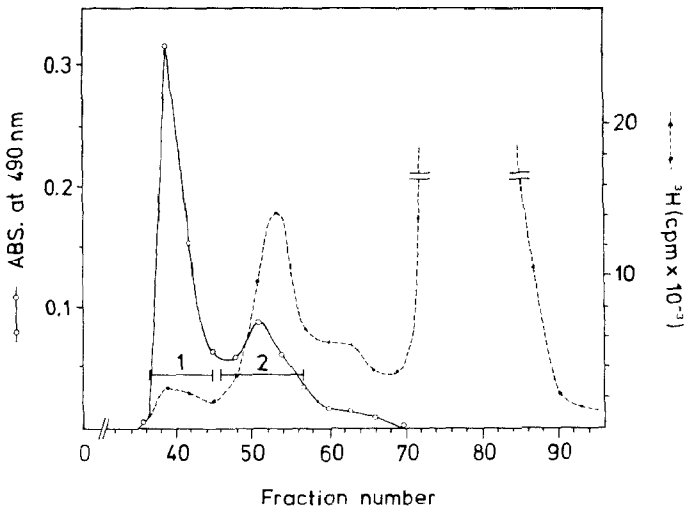


Fig. 1. Elution of the alkaline-borohydride degraded glycophorins from a column (65 × 2 cm) of Sephadex G-15 with water at 0.3 mL/min. Fractions (3 mL) were assayed for hexose and ^3H. Fractions were combined as indicated by 1 and 2.

alditol derivatives, released by alkaline β-elimination in the presence of $NaBH_4$, were hydrolysed and the monosaccharides obtained were reduced with $NaBD_4$. When analysed by g.l.c.–m.s. as alditol acetates, each monosaccharide showed incorporation of deuterium except GalNAc-ol, thus showing GalNAc to be the only sugar reduced during the β-elimination reaction (Table I). The oligosaccharide-alditols were subjected to methylation analysis before and after desialylation (Table II). Most of the Gal residues were 3-substituted in the untreated oligosaccharide-alditols and were converted into non-reducing terminals by desialylation, suggesting the presence of the Neu5Ac-(2→3)-Gal sequence. The presence of 4-substituted GlcNAc and both 3- and 3,6-substituted GalNAc-ol in the untreated and the desialylated oligosaccharides indicated their substitution by

TABLE I

CARBOHYDRATE COMPOSITION OF THE COMPOUNDS IN FRACTIONS 1 AND 2 IN FIG. 1, EXPRESSED IN MOL PER 100 MOL OF TOTAL SUGAR

Sugar	*Fraction 1*	*Fraction 2*
Fuc	6.9	0
Man	13.3	1.6
Gal	18.6	24.6
GlcNAc	43.3	7.6
GalNAc-ol	1.5	30.5
Neu5Ac	12.5	35.7

TABLE II

PARTIALLY METHYLATED ALDITOL ACETATES OBTAINED FROM FRACTION 2 IN FIG. 1

Parent sugar	*Sialo*	*Asialo*
2,3,4,6-Me-Gal	trace[a]	+
2,4,6-Me-Gal	+	trace
1,4,5,6-Me-GalN(Me)Ac	+	+
1,4,5-Me-GalN(Me)Ac	+	+
3,6-Me-GlcN(Me)Ac	+	+

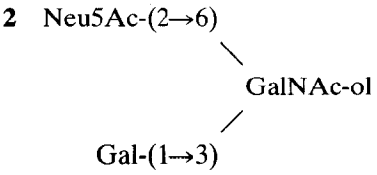
[a]<0.05 if 2,4,6-Me-Gal or 2,3,4,6-Me-Gal is set to 1.0.

residues other than sialic acid. However, GalNAc-ol was substituted partially at position 6 by sialic acid residues, since, after desialylation, the amount of 3,6-disubstituted GalNAc-ol decreased and the amount of 3-substituted GalNAc-ol increased relative to that of the 4-substituted GlcNAc.

The mixture of oligosaccharide-alditols was analysed by f.a.b.-m.s. after methylation (Fig. 2). The assignment of the ions *m/z* 873, 1235, and 1684 to $[M + 1]^+$ was confirmed by the addition of sodium iodide to the matrix which caused each of these ions to shift to $[M + 23]^+$; no other shifts were observed. Information about the monosaccharide sequence was obtained from the ions formed by cleavage of the glycosidic linkages. The primary and secondary fragments *m/z* 376 and 344 indicated non-reducing terminal Neu5Ac residues. The ion *m/z* 580 defined the sequence Neu5Ac–Hex and that at *m/z* 825 the sequence Neu5Ac–Hex–HexNAc. These data together with the results of the sugar and methylation analyses indicated the following structures:

1 Neu5Ac-(2→3)-Gal-(1→3)-GalNAc-ol $[M + 1]^+$ 873

2



$[M + 1]^+$ 873

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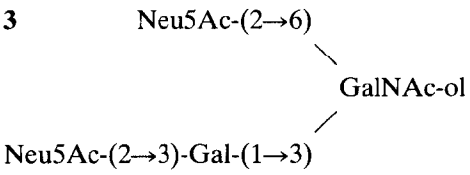


$[M + 1]^+$ 1235

4 Neu5Ac-(2→3)-Gal-(1→4)-GlcNAc-(1→6,3)
GalNAc-ol [M + 1]$^+$ 1684
Neu5Ac-(2→3)-Gal-(1→3,6)

According to the methylation analysis data, the mixture of oligosaccharide-alditols should contain a minor component with non-reducing terminal Gal. The only structure compatible with a non-reducing terminal Gal and the [M + 1]$^+$ value of 873 is **2**.

The methylation analyses of the sialylated and desialylated oligosaccharide-alditols served to determine the positions of substitution of the 3,6-disubstituted GalNAc-ol in structures **2** and **3**. In order to determine the positions of substitution of the GalNAc-ol in structure **4**, a recently developed method was employed[10]. The mixture of oligosaccharide-alditols (fraction 2 from the Sephadex G-15 column) was oxidised with periodate, reduced ($NaBD_4$), acetylated, and analysed by f.a.b.-m.s. According to the methylation analysis data, the GalNAc-ol is 3- and 3,6-substituted and should therefore be cleaved by periodate to form oligosaccharides having either ethylene glycol or 2-acetamido-2-deoxythreitol as aglycons. The f.a.b. mass spectrum of the products formed is shown in Fig. 3. The ions *m/z* 362, 793, and 937 were assigned to [M + 1]$^+$, which was confirmed for the two latter ions by the [M + 23]$^+$ ions of *m/z* 815 and 959 formed on the addition of sodium iodide to the matrix. These ions, however, were 60 mass units less than expected considering oxidation of all periodate-susceptible residues. Furthermore, the primary fragment that should be formed by cleavage of the glycosidic linkage of the oxidised sialic acid was absent, probably due to lactone formation by sialic acid in the acetylated oligosaccharides. Investigation of the model compound α-Neu5Ac-(2→3)-β-Gal-(1→4)-Glc confirmed that a lactone is formed during acetylation and the ^{1}H-n.m.r. and f.a.b.-m.s. data revealed that this involved HO-2 of the Gal residue (data not shown).

The [M + 1]$^+$ ion with *m/z* 362 is consistent with an oxidised sialic acid linked to an ethylene glycol residue (Fig. 3), which showed that one of the structural elements of the oligosaccharide-alditols studied was Neu5Ac-(2→6)-GalNAc-ol (**2** and **3**). The primary fragment with *m/z* 545 corresponded to a Neu5Ac-(2→3)-Gal sequence since a 3-substituted hexosyl residue is resistant to periodate oxidation.

The formation of a fragment with *m/z* 231, corresponding to a 2-acetamido-2-deoxythreitol residue, combined with the sequence ion with *m/z* 545 and a [M + 1]$^+$ ion with *m/z* 793, indicated the presence of the structural element Neu5Ac-(2→3)-Gal-(1→3)-GalNAc-ol (**1**, **3**, and **4**). This is the structure of the linear trisaccharide-alditol **1**, which also was determined from the f.a.b. mass spectrum and the chemical analyses. The sequence ions with *m/z* 545 and 832, and a [M + 1]$^+$ ion with *m/z* 937, indicated the structural element Neu5Ac-(2→3)-Gal-

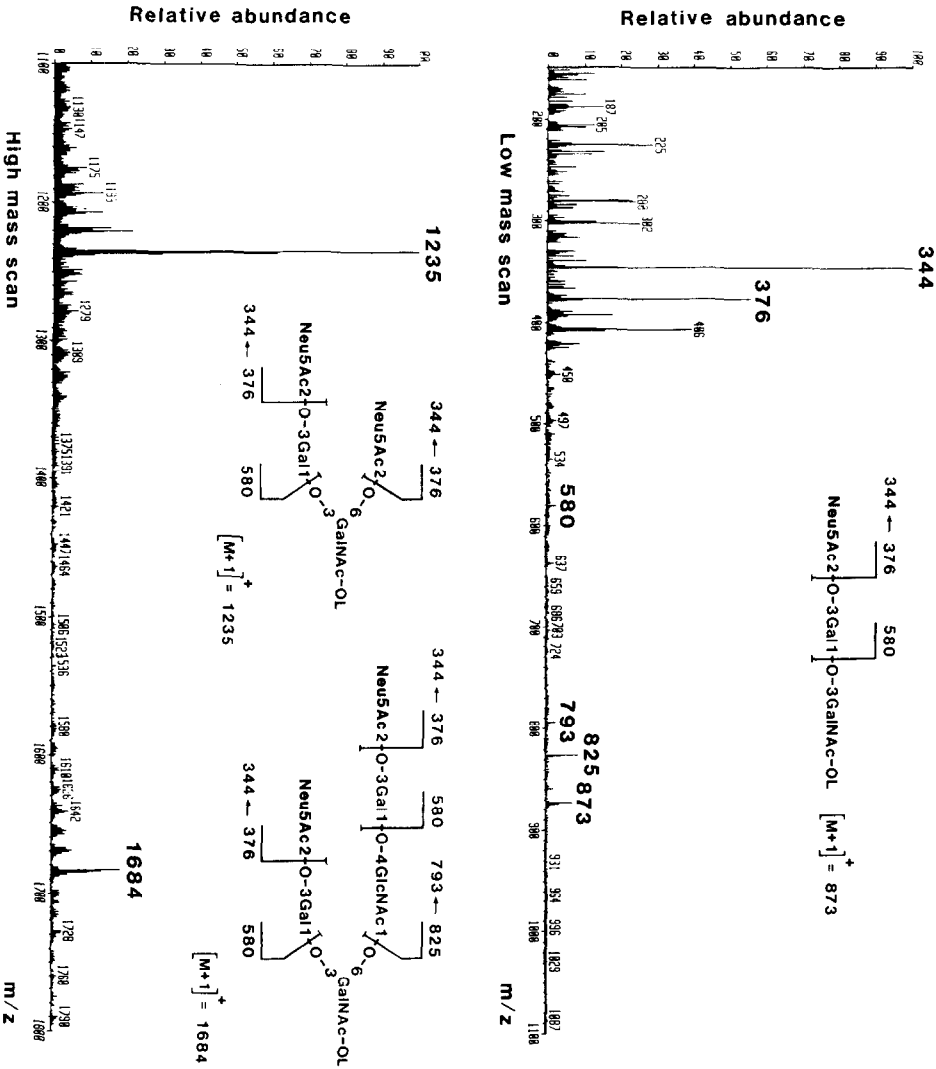


Fig. 2. F.a.b.-mass spectrum of the mixture of methylated alditols derived from the O-linked oligosaccharides.

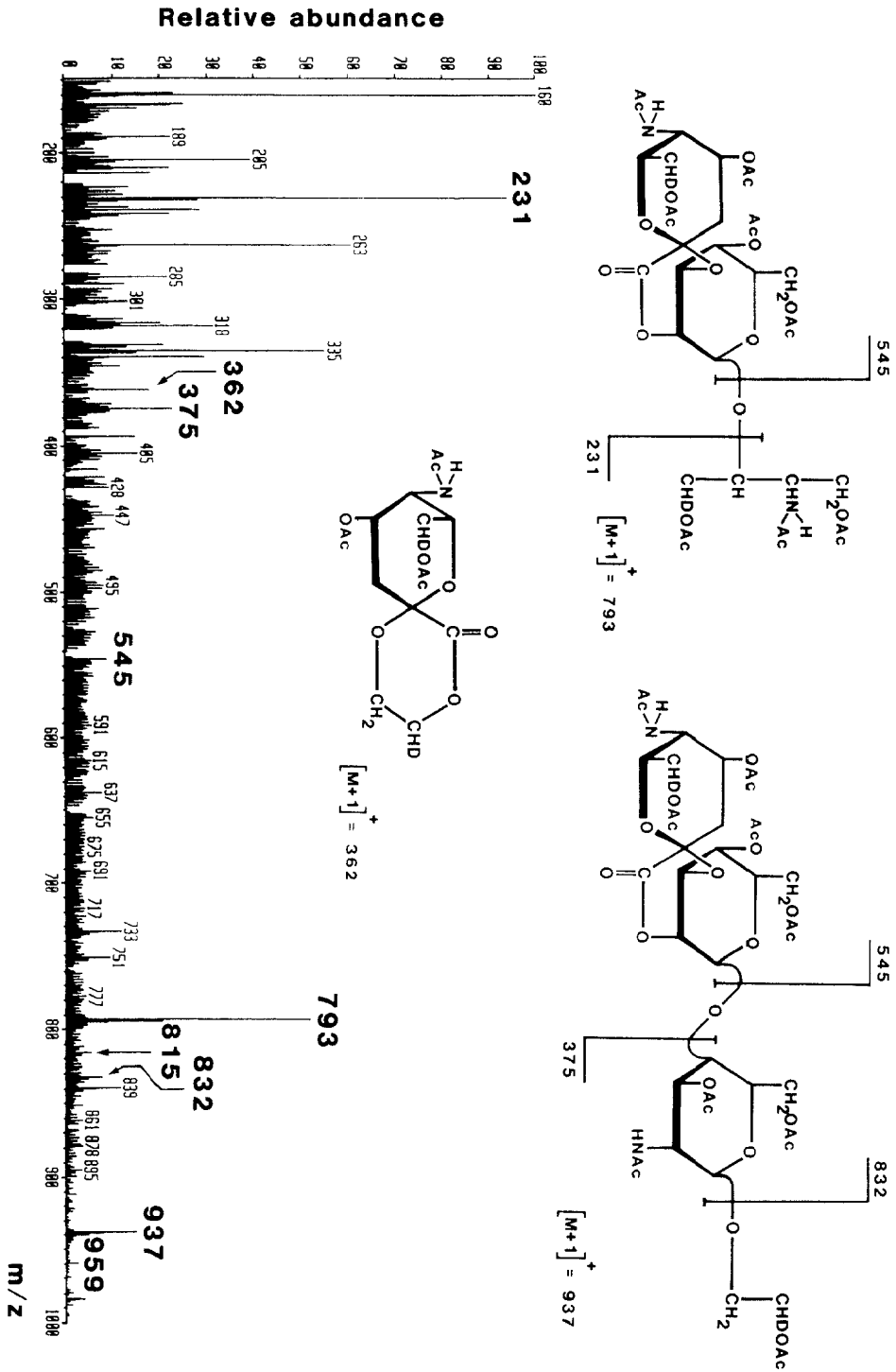
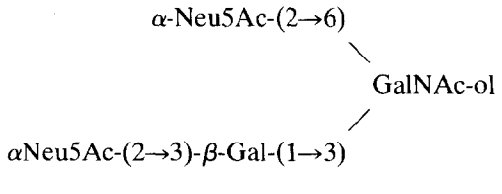


Fig. 3. F.a.b.-mass spectrum of the products formed after periodate oxidation, reduction ($NaBD_4$), and acetylation of the mixture of the alditols derived from the O-linked oligosaccharides.

(1→4)-GlcNAcl-ethylene glycol, showing that the Neu5Ac-(2→3)-Gal-(1→4)-GlcNAc sequence is linked to position 6 of the GalNAc-ol (**4**). Structure **2**, after periodate oxidation, $NaBD_4$-reduction, and acetylation, should give a $[M + 1]^+$ ion with m/z 511 corresponding to a structural element of Gal-(1→3)-GalNAc-ol.

However, this ion was of too low abundance to be unambigously identified. Therefore, the branched structures must be



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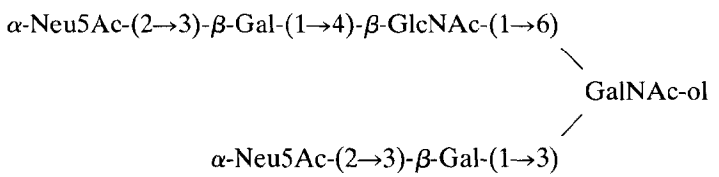

TABLE III

^{1}H-N.M.R. DATA FOR THE MIXTURE OF OLIGOSACCHARIDE-ALDITOLS **1**, **3**, AND **4**

Residue	*Reporter*	*Chemical shifts*[a]		
		1	**3**	**4**
GalNAc-ol	H-2	4.390	4.378	4.390
	H-3	4.07	4.068	[b]
	H-4			[b]
	H-5	4.189	4.244	[b]
	NAc	2.045	2.042	2.065
β-Gal-(1→3)	H-1	4.545	4.543	4.529
	H-3	4.117	4.117	4.117
β-GlcNAc-(1→6)	H-1			4.552
	H-6			4.004
	NAc			2.063
β-Gal-(1→4)	H-1			4.546
	H-4			3.956
α-Neu5Ac-(2→3)	H-3*a*	1.801	1.801	[b]
	H-3*e*	2.774	2.774	[b]
	NAc	2.031	2.031	[b]
α-Neu5Ac-(2→6)	H-3*a*		1.693	
	H-3*e*		2.725	
	NAc		2.032	

[a]In p.p.m. relative to internal acetone (2.225 p.p.m.). [b]Not determined due to overlapping signals and/or low signal-to-noise.

The ^{1}H-n.m.r. data for the reporter groups of the mixture of oligosaccharide-alditols are listed in Table III. These types of structures have been investigated thoroughly by ^{1}H-n.m.r. spectroscopy[20,21] and data which accorded with those reported were obtained. A weak signal at 4.473 p.p.m. showed that a minor component of the oligosaccharide-alditols contained non-reducing terminal Gal, but no further information about the structure could be inferred from the n.m.r. spectrum.

DISCUSSION

The use of inbred mice as experimental animals has been increased greatly by the availability of monoclonal antibodies. Many such antibodies directed against carbohydrate antigens (*e.g.*, blood-group determinants, tumor markers) have been obtained. Therefore, it is important to know the carbohydrate structures of mouse cells. Since little is known about the surface glycoconjugate structures of mouse erythrocytes, studies of oligosaccharide chains of mouse glycophorins were undertaken. BALB/c mice were chosen, since they are frequently used in biological experiments. A strategy to elucidate the structure of the alditols derived from the O-linked oligosaccharides without prior separation was used. Structural analysis of mixtures of oligosaccharides, by f.a.b.-m.s., has been reported[22].

Methylation analysis and f.a.b.-m.s. of the untreated oligosaccharide-alditols gave information about their sizes and sequences. The final determination of the pattern of substitution of the branched residue in the hexasaccharide was carried out using a variation of the recently developed method[10] in which the products of periodate oxidation, reduction, and methylation were analysed by f.a.b.-m.s. Acetylation was used instead of a methylation since the products are less volatile and have higher molecular weights, which makes them more amenable to f.a.b.-m.s.

Analysis of the acetylated products showed the absence of primary fragments that should be formed by cleavage of oxidised sialic acid residues. Moreover, the products containing sialic acid had molecular weights that were 60 mass units lower than predicted, and lactone formation had occurred. It has been shown by ^{1}H-n.m.r. studies that HO-2 of the D-galactose in G_{M3} ganglioside was esterified by sialic acid[23], and a similar result was found here for the acetylated model compound α-Neu5Ac-(2→3)-β-Gal-(1→4)-Glc.

F.a.b.-m.s. and n.m.r. analyses showed that the O-linked oligosaccharide chains in BALB/c mouse erythrocyte glycophorins were converted into the trisaccharide-alditol α-Neu5Ac-(2→3)-β-Gal-(1→3)-GalNAc-ol, the tetrasaccharide-alditol α-Neu5Ac-(2→3)-β-Gal-(1→3)-[α-Neu5Ac-(2→6)]-GalNAc-ol, and the hexasaccharide-alditol α-Neu5Ac-(2→3)-β-Gal-(1→3)-[α-Neu5Ac-(2→3)-β-Gal-(1→4)-β-GlcNAc-(1→6)]-GalNAc-ol. Each of these structures contains Neu5Ac, but BALB/c mouse erythrocyte glycophorins contain mostly 9-*O*-acetyl-*N*-acetylneuraminic acid[7], which was *O*-deacetylated during the preparation of the above derivatives.

The methylation analysis and n.m.r. data of the mixture of oligosaccharide-alditols showed weak evidence for a component containing non-reducing terminal Gal, for which structure **2** was suggested but no supporting data were obtained.

Desialylation of the precursors of the above tri- and tetra-saccharide-alditol derivatives should expose the β-Gal-(1→3)-GalNAc unit [Thomsen–Friedenreich (TF) receptor[24]], which can be recognised using anti-TF antibodies and some lectins. In fact, the desialylated glycophorins of BALB/c mouse erythrocytes reacted with peanut agglutinin and anti-TF monoclonal antibody, as do human, horse, and dog erythrocyte glycophorins[25]. The hexasaccharide precursor of **4** is present in rat glycophorin[26], platelets[27,28], tumor cells[29–32], and fetuin[33]. Similar O-linked oligosaccharides, elongated at C-6 of the GalNAc residue, may also be present as trace components in human glycophorins, where their amount is increased in some variant molecules[1], but the structure of these oligosaccharides has not been fully elucidated. Thus, the *O*-acetylated sialic acid(s) and the GlcNAc-containing O-linked hexasaccharide precursor of **4** may be considered as characteristic features of the BALB/c mouse erythrocyte glycophorins. The availability of inbred strains of mice allows a comparison of the erythrocyte glycophorins from various strains.

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