Carbohydrate Research, 221 (1991) 191–208
Elsevier Science Publishers B.V., Amsterdam

Characterisation of minor tetra- to hepta-saccharides O-linked to human meconium glycoproteins by t.l.c.–m.s. microsequencing of neoglycolipid derivatives in conjunction with conventional m.s. and ^{1}H-n.m.r. spectroscopy

Alexander M. Lawson[a], Elizabeth F. Hounsell[b], Mark S. Stoll[b], James Feeney[c], Wengang Chai[a], Jerzy R. Rosankiewicz[a], and Ten Feizi[b].

Sections of Clinical Mass Spectrometry[a] and Glycoconjugates[b], Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ (Great Britain) and M.R.C. Biomedical N.M.R. Centre[c], National Institute for Medical Research, Mill Hill, London NW7 1AA (Great Britain)

(Receied May 4th, 1991; accepted for publication June 27th, 1991)

ABSTRACT

As part of the establishment of a data base for core and backbone sequences of O-linked oligosaccharides of human meconium glycoproteins, the minor tetra- to hepta-saccharides released by mild-acid treatment of blood group H-active glycoproteins have been studied. These oligosaccharides are heterogeneous and difficult to isolate, and a t.l.c.–m.s. microsequencing procedure has been applied to the neoglycolipid derivatives, in conjunction with ^{1}H-n.m.r. spectroscopy, methylation analysis, and mass spectrometry (m.s.) of native and methylated oligosaccharides. Among an array of oligosaccharides characterised are those having the branched β-GlcNAc-(1→6)[β-Gal-(1→3)]-GalNAcol core, and others with the following linear sequences not characterised previously from this source: β-Gal-(1→3)-β-GlcNAc-(1→3)-β-Gal-(1→3)-GalNAcol, β-Gal-(1→4)-β-GlcNAc-(1→3)-β-Gal-(1→3)-GalNAcol, α-GalNAc-(1→3)-β-Gal-(1→3/4)-β-GlcNAc-(1→3)-GalNAcol, β-GlcNAc-(1→3)-β-Gal-(1→3/4)-β-GlcNAc-(1→3)-GalNAcol, β-Gal-(1→3/4)-β-GlcNAc-(1→3)-β-Gal-(1→3/4)-β-GlcNAc-(1→3)-β-Gal-(1→3)-GalNAcol, and β-GlcNAc-(1→3)-β-Gal-(1→3/4)-β-GlcNAc-(1→3)-β-Gal-(1→3/4)-β-GlcNAc-(1→3)-GalNAcol.

INTRODUCTION

Meconium, present in the gastrointestinal tract of the neonate, is a rich source of mucin type glycoproteins which express an array of carbohydrate differentiation antigens recognised by monoclonal antibodies[1]. The present report is one of a series[2–5] principally aimed at elucidating the structure of core and backbone regions of the O-linked oligosaccharides on these glycoproteins. Combined blood-group H-active meconium samples from neonates were digested with pronase, depleted of minor components with Ii antigenic activities by immunoaffinity chromatography, and subjected to mild acid hydrolysis to release fucose and sialic acid[2]. The oligosaccharides subsequently liberated on treatment with alkali and borohydride were fractionated and purified by chromatography on Bio-Gel P4 and h.p.l.c. The major mono- to hexa-saccharides were characterised[2–4] by coventional m.s. and ^{1}H-n.m.r. spectroscopy. For oligosaccharides that were available in amounts too small for complete elucidation of their structures by these techniques, a microscale sequencing procedure based on t.l.c.–m.s. of the derived

0008-6215/91/$03.50

neoglycolipids was introduced[5]. We now report the application of this procedure, in conjunction with m.s. and ^{1}H-n.m.r. spectroscopy, in order to characterise minor, heterogeneous, oligosaccharide populations among the tetra- to hepta-saccharides.

EXPERIMENTAL

Materials. — H.p.t.l.c. plates (aluminium-backed 5-μm silica, Merck) were purchased from B.D.H., C_{18} Bond Elut columns (10 mg in 1 mL) from Jones Chromatography, and sodium cyanoborohydride, phosphatidylethanolamine dipalmitoate, primulin, and imidazole from Sigma. All solvents were of Analytical Reagent grade.

Oligosaccharides. — Oligosaccharides were released from meconium glycoproteins and fractionated on Bio-Gel P4 as described[2]. The fractions M and J were eluted at positions corresponding to 8–9 and 11–12 glucose residues, respectively, and flanked the major fraction K described earlier[4]. Fraction M amounted to 5% of the total oligosaccharides released and fraction J to 8%.

H.p.l.c. — Fractions M and J were subjected to reverse-phase (r.p.) h.p.l.c. on a column (250 × 4 mm) of ODS-Hypersil (Shandon Southern Products) by elution with water, using an SP8700 solvent delivery system, an SP8400 variable wavelength detector at 208 nm, and an SP4100 computing integrator (Spectra Physics) (Figs. 1 and 2). The products in the major carhohydrate peaks in subfractions M2, M3, and M5 were purified further by h.p.l.c. on APS-Hypersil (Shandon), using a gradient of acetonitrile–water 65:35 → 60:40 (results not shown). Fractions J2–J6 were then subjected to r.p.-h.p.l.c. to give subfractions J2(2)–J6(7) as defined in Fig. 2.

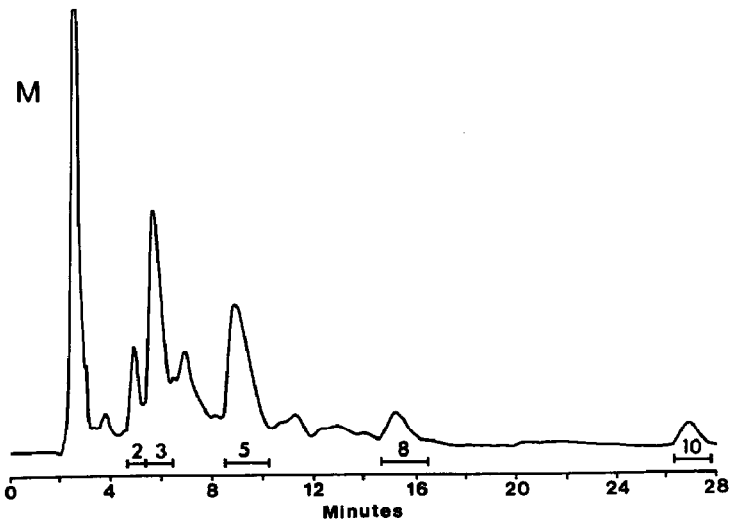


Fig. 1. R.p.-h.p.l.c. of fraction M on an ODS-Hypersil column. Fractions 2, 3, 5, 8, and 10 were taken for further purification on APS-Hypersil.

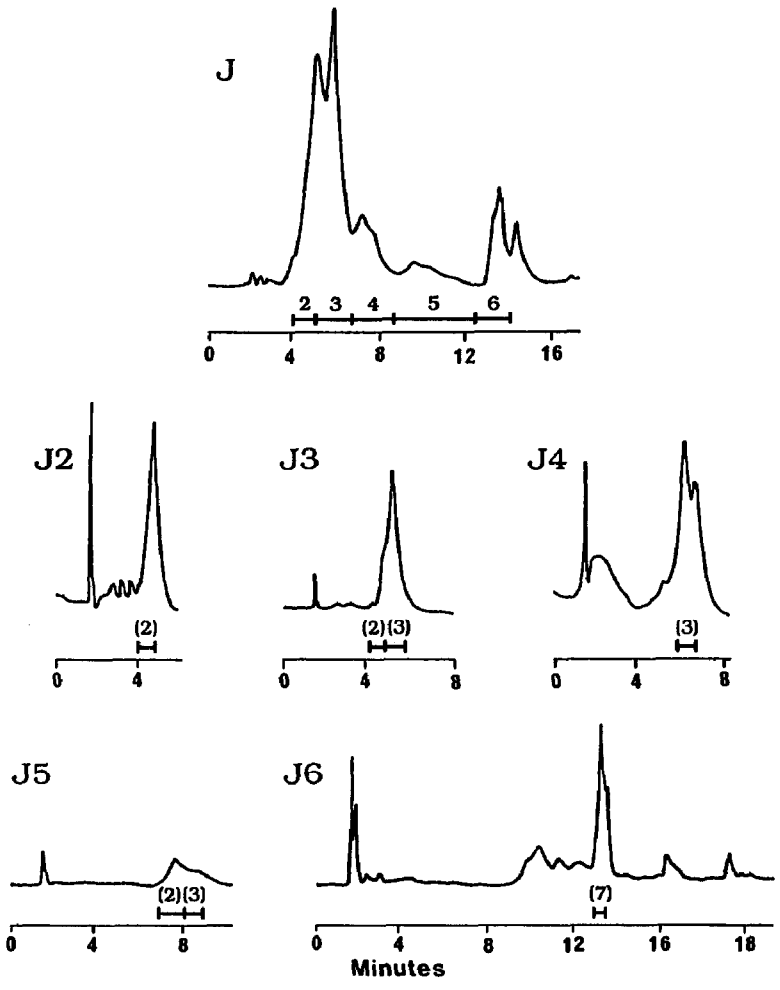


Fig. 2. R.p.-h.p.l.c. of fraction J on ODS-Hypersil and rechromatography of subfractions 2–6 on ODS-Hypersil.

Methylation analysis. — Oligosaccharides were permethylated by the method of Ciucanu and Kerek[6], and the partially methylated alditol acetates were obtained by established procedures[4].

Conversion[5] of mucin alditols into neoglycolipids for t.l.c.–m.s. — Briefly, a mild periodate oxidation step (which cleaved the C-4–C-5 bond of the core 2-acetamido-2-deoxygalactitol) was followed by a reductive amination reaction to conjugate the resulting oligosaccharide fragments to phosphatidylethanolamine dipalmitoate. The mixture was then desalted on a C_{18} Bond Elut column and subjected to t.l.c. using chloroform–methanol–water (130:50:9). Oligosaccharide sequences 3- and 6-linked to GalNAcol can be distinguished by the characteristic *m/z* values (835 and 734, respectively) of ions from the derivatives designated 3-OX and 6-OY which contain the fragments derived from GalNAcol, where X is –CH[CH(NHAc)CH_2OH]CH_2–lipid

and Y is $-CH_2CH_2-$lipid, and the lipid moiety is $-NHCH_2CH_2OP(=O)(-OH)OCH_2CH[-OCO(CH_2)_{14}CH_3]CH_2OCO(CH_2)_{14}CH_3$.

Liquid secondary-ion mass spectrometry[4] *(l.s.i.-m.s.).* — A VG Analytical ZAB2-E mass spectrometer was used with a caesium ion gun (35 keV, 0.5 μA), and mass spectra (8 keV accelerating voltage and 1500 resolving power) were obtained from samples (0.2–1 nmol, dissolved in methanol) in a 1:1 glycerol–thioglycerol matrix. Spectra of the carbohydrates and their methylated derivatives were acquired in the negative and positive modes, respectively.

Negative-ion mass spectra of neoglycolipids were obtained by direct t.l.c.–l.s.i.-m.s.[5,7], using a 2:2:1 matrix of diethanolamine–tetramethylurea–*m*-nitrobenzyl alcohol (3 μL) and added chloroform–methanol–water (25:25:8, 2 μL).

^{1}H-N.m.r. spectroscopy. — 500-MHz spectra were obtained with a Bruker AM500 spectrometer operating in the Fourier-transform mode and equipped with an Aspect 3000 computer. Chemical shifts were referenced to internal acetone (2.225 p.p.m. at 22°), which in turn was referenced to sodium 4,4-dimethyl-4-silapentane-1-sulphonate. Interpretation of the spectra was carried out in combination with an interactive computer programme[8].

RESULTS

M fractions (see Table I). — The major oligosaccharide structure in fraction M2 (Fig. 1) was shown by ^{1}H-n.m.r. spectroscopy (Table II) and methylation analysis (Table III) to be a tetrasaccharide that had been characterised[2] in the adjacent Bio-Gel fraction N.

Fraction M3 consisted of two major tetrasaccharides (Table I) having ^{1}H-n.m.r. spectra (Table II) consistent with those of documented structures[9–11]. The l.s.i.-mass spectrum of the methylated fraction accorded with this composition (*i.e.* *m/z* 961 for $[M + H]^+$), and methylation analysis (Table III) indicated a 2:3 mixture of the tetrasaccharides having β-Gal-(1→3)-GlcNAc and β-Gal-(1→4)-GlcNAc linkages as suggested by the ^{1}H-n.m.r. data. Additional information on the sequence was obtained by t.l.c.–m.s. microsequencing of the neoglycolipids. The major neoglycolipid band (band 10, Fig. 3) gave an $[M-H]^-$ ion at *m/z* 1362 with fragment ions at *m/z* 1200, 997, and 835 in agreement with the Gal–GlcNAc–Gal sequence 3-linked to the core GalNAcol. Minor components in M3 gave additional bands at positions 1, 2, and 4–6 (Fig. 3) representing oligosaccharides of various sequences shown in Table IV that were either 3- or 6-linked to GalNAcol. The type of acetamido sugar residues in the proposed HexNAc–HexNAc– sequence 6-linked to GalNAcol, with $[M-H]^-$ at *m/z* 1140 (band 5) and fragment ions at *m/z* 937 and 734, could not be assigned, as both terminal GlcNAc and GalNAc were detected as minor constituents by methylation analysis. Neither sequence has been recorded previously on the O-linked chains.

^{1}H-n.m.r. spectroscopy (Table II) indicated the major component of fraction M5 to be a fucosylated analogue (Table I) of the major structure in M2. This sequence has been documented in ovarian cyst[12] and bronchial mucins[9,13]. The l.s.i.-mass spectrum of

TABLE I

The main oligosaccharide structures identified in Fractions M and J

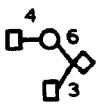
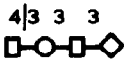
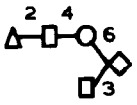
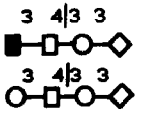
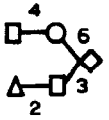
Oligosaccharide	*Structure*
M2	β-Gal-(1→4)-β-GlcNAc-(1→6) GalNAcol β-Gal-(1→3)
M3	β-Gal-(1→3)-β-GlcNAc-(1→3)-β-Gal-(1→3)-GalNAcol β-Gal-(1→4)-β-GlcNAc-(1→3)-β-Gal-(1→3)-GalNAcol
M5	α-Fuc-(1→2)-β-Gal-(1→4)-β-GlcNAc-(1→6) GalNAcol β-Gal-(1→3)
M8	α-GalNAc-(1→3)-β-Gal-(1→3/4)-β-GlcNAc-(1→3)-GalNAcol β-GlcNAc-(1→3)-β-Gal-(1→3/4)-β-GlcNAc-(1→3)-GalNAcol
M10	β-Gal-(1→4)-β-GlcNAc-(1→6) GalNAcol α-Fuc-(1→2)-β-Gal-(1→3)
J2(2)	β-Gal-(1→4)-β-GlcNAc-(1→6) GalNAcol β-Gal-(1→4/3)-β-GlcNAc-(1→3)-β-Gal-(1→3)
J3(2)	Gal-GlcNAc Gal-(→3)-GalNAcol Gal-GlcNAc
J3(3)	β-Gal-(1→4/3)-β-GlcNAc-(1→3)-β-Gal-(1→4)-β-GlcNAc-(1→6) GalNAcol β-Gal-(1→3)
J4(3)	β-Gal-(1→3/4)-β-GlcNAc-(1→3)-β-Gal-(1→3/4)-β-GlcNAc-(1→3)-β-Gal-(1→3)-GalNAcol
J6(7)	β-GlcNAc-(1→3)-β-Gal-(1→3/4)-β-GlcNAc-(1→3)-β-Gal-(1→3/4)-β-GlcNAc-(1→3)-GalNAcol

the methylated fraction contained the fragment-ion doublet *m/z* 638/606 (*i.e.* Fuc–Gal–GlcNAc–), and the results of methylation analysis (Table III) were consistent with this assignment. The branching pattern of the M5 pentasaccharide was also evident from microsequencing of the neoglycolipid derivatives which gave bands 1 and 7 with *m/z* 997 and 1245, respectively (Fig. 3, Table IV). T.l.c.–m.s. of the other component bands (Table IV) indicated the presence of Gal–GlcNAc–Gal–GlcNAc 6-linked to GalNAcol (band 11) and Gal–GlcNAc–(Gal–GlcNAc–)Gal 3-linked to GalNAcol (band 12).

The ^{1}H-n.m.r. data for M8 (Table II) suggested the presence of a mixture of linear isomeric tetrasaccharides containing α-GalNAc or β-GlcNAc and with either a β-Gal-(1→3)-GlcNAc or β-Gal-(1→4)-β-GlcNAc sequence 3-linked to GalNAcol (Table I). The resonances were assigned by comparison with data in the literature for linear trisaccharides having the β-GlcNAc-(1→3)-GalNAcol core[2,9,10]. The l.s.i.-mass spec-

TABLE II

The ^{1}H-n.m.r. chemical shifts (p.p.m. from d.s.s.) of the tetra- to penta-saccharide fraction from meconium glycopeptides.

Residue	*Atom*	*M2*[a] 	*M3* 	*M5* 	*M8* 	*M10* 
GalNAcol[b]	H-2	4.397	4.401	4.395	4.291	4.400
	H-3	4.063	4.052	4.063	4.015	4.084
	H-4	3.461	3.493	3.463	–	3.499
	H-5	4.286	4.188	4.284	4.141	4.273
	NAc	2.067	2.047	2.066	2.039,2.033	2.055
GlcNAc[6]	H-1	4.558		4.535		4.569
	H-6	3.999		–		4.001
	NAc	2.067		2.067		2.055
^{4}GlcNAc[3]	H-1		4.683		4.646	
	NAc		2.043		2.075	
^{3}GlcNAc[3]	H-1		4.704		4.658	
	NAc		2.035		2.065	
GlcNAc[3]	H-1				4.700	
	NAc				–	
Gal[3]	H-1	4.466	4.466	4.465		4.574
(to GalNAcol)	H-2	3.537	3.591	3.562		3.538
	H-4	3.932	4.129	–		–

Gal	H-1	4.470	4.482	4.538	4.515	4.479
	H-2	3.561	3.541	–	–	–
	H-4	–[c]	3.928	–	–	–
Gal	H-1		4.462		4.455	
	H-2		3.541		–	
Fuc	H-1			5.312		5.223
	H-5			4.235		4.267
	CH_3			1.232		1.244
α-GalNAc	H-1				5.077	
	H-2				4.224	
	H-4				4.102	
	H-5				4.203	
	NAc				2.031	

[a] Symbols: ◇, GalNAcol; ○, β-GlcNAc; □, β-Gal; ■, α-GalNAc; △, Fuc. [b] Numbers shown as superscripts refer to the position of linkage. [c] Not determined.

TABLE III

Saccharide composition and linkage assignments from methylation analysis of oligosaccharide fractions[a]

Linkage	*Oligosaccharide fractions*										
	M2	*M3*	*M5*	*M8*	*M10*	*J2(2)*	*J3(2)*	*J3(3)*	*J4(3)*	*J5(3)*	*J6(7)*
	Relative peak intensities										
Fuc-(1→)	–	–	1	+	1	+	–	–	–	0.5	+
Gal-(1→)	2	1	1	+	1	1	2	2	1	1.7	1
(→4)-Gal-(1→)	–	+	–	+	–	0.5	+	–	+	+	+
(→3)-Gal-(1→)	–	1	–	1	–	1	0.8	1	2	2	1
(→2)-Gal-(1→)	–	–	1	–	1	–	–	–	–	+	0.5
(→6)-Gal-(1→)	–	+	–	–	–	+	+	+	–	+	+
(→3/2)-Gal-(1→)	–	–	–	–	–	0.3	–	–	+	0.4	–
(→6/3)-Gal-(1→)	–	–	–	–	–	–	+	0.5	+	+	–
(→3)-GalNAcol	–	1	–	1	–	–	+	0.5	1	–	+
(→6/3)-GalNAcol	1	+	1	–	1	1	1	0.5	+	0.5	+
GlcNAc-(1→)	–	+	+	0.4	–	+	+	0.5	+	0.5	+
GalNAc-(1→)	–	–	–	0.6	–	–	–	–	–	–	–
(→4)-GlcNAc-(1→)	1	0.4	1	0.3	1	1	1.5	1.5	0.7	1.5	2
(→3)-GlcNAc-(1→)	–	0.6	–	0.7	–	+	+	0.8	1.3	1.8	2

[a] Symbols: +, partially methylated alditol acetates arising from minor components; –, not detected.

TABLE IV

L.s.i.-m.s. data and proposed monosaccharide sequences of neoglycolipids derived from oligosaccharide fractions M3, M5, M8, and M10

T.l.c. position	*Structure*[a]	*Mass of [M − H]⁻ ion*[b]	*Mass of fragment ions*	*Oligosaccharide-alditol fraction*			
1	Gal-3-OX	997	835	M3	M5		
2	GlcNAc-6-OY	937	835	M3			
3	Fuc-Gal-3-OX	1143	997, 835				M10
4	Gal-GlcNAc-3-OX	1200	1038, 835	M3			
5	HexNAc-HexNAc-6-OY	1140	937, 734	M3			
6	Gal-GlcNAc-6-OY	1099	937, 734	M3			M10
7	Fuc-Gal-GlcNAc-6-OY	1245	1099, 937, 734		M5		
8	GalNAc-Gal-GlcNAc-3-OX	1403	1200, 1038, 835			M8	
9	GlcNAc-Gal-GlcNAc-3-OX	1403	1200, 1038, 835			M8	
10	Gal-GlcNAc-Gal-3-OX	1362	1200, 997, 835	M3			
11	Gal-GlcNAc-Gal-GlcNAc-6-OY	1464	1302, 1099, 937, 734		M5		
12	Gal-GlcNAc Gal-3-OX Gal-GlcNAc	1727	1565, 1362, 835		M5		

[a] X = $CH[CH(NHAc)CH_2OH]CH_2$-lipid, Y = CH_2CH_2-lipid where lipid = $-NHCH_2CH_2OP(=O)(-OH)OCH_2CH[-OCO(CH_2)_{14}CH_3]CH_2OCO(CH_2)_{14}CH_3$.
[b] Mass values rounded down to nearest nominal mass.

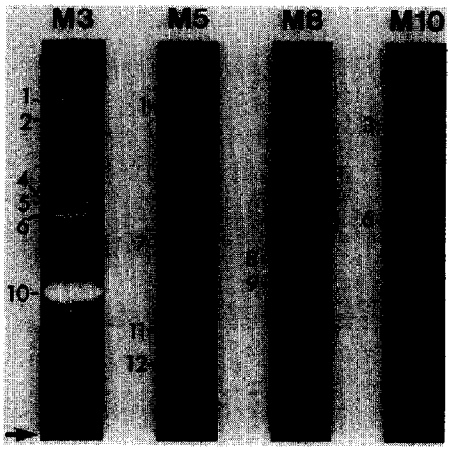


Fig. 3. Primulin-stained thin-layer chromatogram of the neoglycolipids derived from oligosaccharide fractions M3, M5, M8, and M10. Bands at positions 1–12 were analysed by l.s.i.-m.s. and the sequences assigned are listed in Table IV; the arrow indicates the origin.

trum of methylated fraction M8 contained a peak for $[M+H]^+$ at *m/z* 1002 with fragment ions at *m/z* 709 and 260/228 reflecting the GlcNAc–Gal–GlcNAc sequence. Methylation analysis (Table III) indicated that GalNAc-(1→ and GlcNAc-(1→ were present in a ratio 3:2 and →3)-GlcNAc and →4)-GlcNAc were present in a ratio 7:3. The proposed linear tetrasaccharide sequences were confirmed by t.l.c.–m.s. microsequencing of the neoglycolipid products in bands 8 and 9 (Fig. 3), the ratios of the intensities of which were ~2:1. Each product gave an $[M-H]^-$ at *m/z* 1403 and identical fragment ions (Table IV). The difference in R_F values between the two bands reflects the composition, linkage, and anomeric configuration. The faster elution of the sequence with α configuration was expected[5].

Fraction M10 gave a ^{1}H-n.m.r. spectrum (Table II) indicative of a second fucosylated analogue of M2, isomeric with the major component of M5 (Table I), as documented for oligosaccharides of meconium glycopeptides[14] and of ovarian cyst[12] and bronchial[9,13] mucins. The l.s.i.-mass spectrum of the methylated oligosaccharide contained a peak for $[M + H]^+$ at *m/z* 1135, which confirmed the composition, and the fragment-ion doublet *m/z* 638/606 indicating the Fuc–Gal–GlcNAc sequence. T.l.c.–m.s. microsequencing of the two major neoglycolipid products of M10 (bands 3 and 6, Fig. 3) with $[M-H]^-$ ions of *m/z* 1143 and 1099, respectively, and their fragment ions (Table IV), supported the assignment of structure.

J fractions (see Table I). — Due to the multiplicity of oligosaccharides in these fractions and their relatively small amounts, it was difficult to make unambiguous assignments of structure by ^{1}H-n.m.r. spectroscopy and methylation analysis, although some key information was obtained. The saccharide compositions of the oligosaccharides in the J subfractions were deduced from their l.s.i.-mass spectra and those of their methylated derivatives (Table V), and detailed sequence and branching assignment of 3- and 6-linked chains to core GalNAcol was made by t.l.c.–m.s. microsequencing of the neoglycolipid derivatives (Fig. 4 and Table VI).

TABLE V

L.s.i.-m.s. data and deduced saccharide compositions of J fractions

	Native	*Permethylated*	*Oligosaccharide composition*
Fraction	*$[M-H]^-$ ions*	*$[M+H]^+$ ions*	
J2(2)	952	1206	Hex_2–$HexNAc_2$–HexNAcol
	1098	1380	Fuc–Hex_2–$HexNAc_2$–HexNAcol
	1114	1410	Hex_3–$HexNAc_2$–HexNAcol
	1260	1584	Fuc–Hex_3–$HexNAc_2$–HexNAcol (minor)
J3(2)	952	1206	Hex_2–$HexNAc_2$–HexNAcol
	1114	1410	Hex_3–$HexNAc_2$–HexNAcol
	1098	1380	Fuc–Hex_2–$HexNAc_2$–HexNAcol
J3(3)	1114	1410	Hex_3–$HexNAc_2$–HexNAcol
	952	1206	Hex_2–$HexNAc_2$–HexNAcol (minor)
	749	961	Hex_2–HexNAc–HexNAcol (minor)
J4(3)	1114	1410	Hex_3–$HexNAc_2$–HexNAcol
	952	1206	Hex_2–$HexNAc_2$–HexNAcol (minor)
J5(2)	1276	1614	Hex_4–$HexNAc_2$–HexNAcol
	1260	1584	Fuc–Hex_3–$HexNAc_2$–HexNAcol
	1098	1380	Fuc–Hex_2–$HexNAc_2$–HexNAcol (minor)
	1114	1410	Hex_3–$HexNAc_2$–HexNAcol (minor)
J5(3)	1098	1380	Fuc–Hex_2–$HexNAc_2$–HexNAcol
	1114	1410	Hex_3–$HexNAc_2$–HexNAcol
	1155	1451	Hex_2–$HexNAc_3$–HexNAcol
	1260	1584	Fuc–Hex_3–$HexNAc_2$–HexNAcol
	1276	1614	Hex_4–$HexNAc_2$–HexNAcol
J6(7)	1155	1451	Hex_2–$HexNAc_3$–HexNAcol
	1098	1380	Fuc–Hex_2–$HexNAc_2$–HexNAcol (minor)
	1260	1584	Fuc–Hex_3–$HexNAc_2$–HexNAcol (minor)

L.s.i.-m.s. and ^{1}H-n.m.r. spectroscopy (Tables V and VII) showed that fraction J2(2) contained a complex mixture of penta- to hepta-saccharides. The data were consistent with the major components being those documented[4] in an adjoining fraction [K3(4)] with the structures

$$\begin{matrix} & & \beta\text{-Gal-}(1\rightarrow4)\text{-}\beta\text{-GlcNAc-}(1\rightarrow6) \\ \beta\text{-Gal-}(1\rightarrow3/4)\text{-}\beta\text{-GlcNAc-}(1\rightarrow3)\text{-}\beta\text{-Gal-}(1\rightarrow3) & & \end{matrix} \diagdown \text{GalNAcol.}$$

The neoglycolipids in bands 3 and 6 (Table VI), produced from J2(2), were consistent with the presence of these oligosaccharides. Other structures suggested from the l.s.i.-mass spectra of native and methylated J2(2) (Table V) were also apparent from the

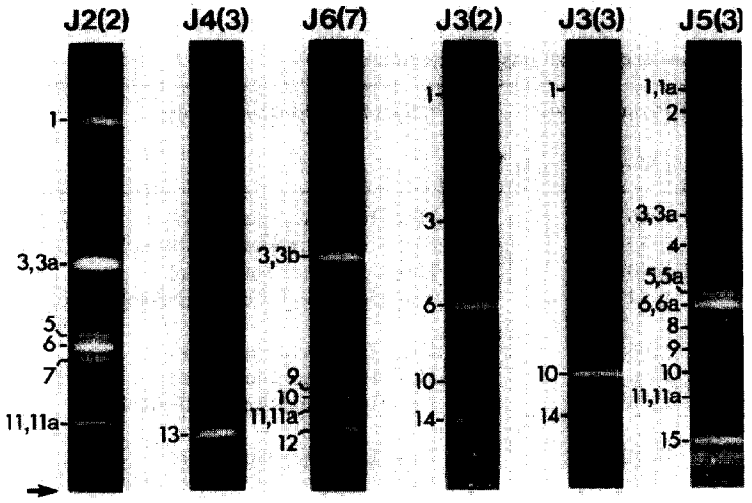
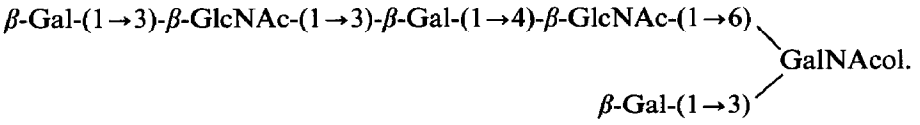


Fig. 4. Primulin-stained thin-layer chromatogram of the neoglycolipids derived from oligosaccharide fractions J2(2), J3(2), J3(3), J4(3), J5(3), and J6(7). The bands 1–15 were analysed by l.s.i.-m.s. and the sequences assigned are listed in Table VI; the arrow indicates the origin. Fractions J2(2), J4(3), and J6(7) were chromatographed on a separate occasion from fractions J3(2), J3(3), and J5(3).

analysis of the neoglycolipid product bands 1, 7, and 11/11a (Fig. 4) with respective $[M-H]^-$ ions at *m/z* 997, 1448, and 1711/1610 and the fragment ions shown in Table VI. Further evidence for these sequences was obtained from fragment ions in the l.s.i.-mass spectra of the methylated oligosaccharides including *m/z* 1087 (Fuc–Gal_2–$GlcNAc_2$), 883 (Fuc–Gal–$GlcNAc_2$), and 464 (Gal–GlcNAc).

^{1}H-N.m.r. spectroscopy and l.s.i.-m.s. indicated that fraction J3(2) contained some of the components identified in J2(2) and in the subsequent fraction J3(3) (Tables V and VII). The microsequencing of the neoglycolipid products (Fig. 4) was consistent with this interpretation and showed bands 1, 3, and 6 in common with J2(2), and bands 1, 10, and 14 in common with J3(3). L.s.i.-m.s. of band 14 from J3(3) indicated the presence of a hexasaccharide with the branched structure Gal–GlcNAc–(Gal–GalNAc–)Gal–(1→3)–GalNAcol as described[4].

The more homogeneous nature of fraction J3(3) enabled the major component to be identified by ^{1}H-n.m.r. spectroscopy (Table VII) as the hexasaccharide characterised[4] in an adjoining fraction [K4(3)].



L.s.i.-m.s. of the oligosaccharide and its methylated derivative and t.l.c.-m.s. microsequencing of bands 1 and 10 (Fig. 4) gave results that were in agreement with this assignment. Additional signals in the ^{1}H-n.m.r. spectrum could not rule out the possibil-

TABLE VI

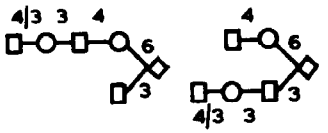
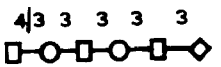
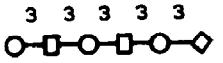
L.s.i.-m.s. data and proposed monosaccharide sequences of neoglycolipids derived from oligosaccharide fractions J2(2), J3(2), J3(3), J4(3), J5(3), and J6(7).

T.l.c. position	*Structure*[a]	*Mass of $[M-H]^-$ ion (Da)*[b]	*Mass of fragment ions (Da)*	*Oligosaccharide-alditol fraction*					
1	Gal-3-OX	997	835	2(2)	3(2)	3(3)		5(3)	
1a	GlcNAc-3-OX	1038	835					5(3)	
2	GlcNAc-6-OY	937	734					5(3)	
3	Gal–GlcNAc-6-OY	1099	937, 934	2(2)	3(2)	3(3)		5(3)	6(7)
3a	GlcNAc–Gal-3-OX	1200	997, 835	2(2)				5(3)	
3b	Fuc–Gal–GlcNAc-3-OX	1346	1200, 1038, 835						6(7)
4	Fuc–Gal–GlcNAc-6-OY	1245	1099, 937, 734					5(3)	
5	Gal–GlcNAc–Gal-3-OX	1362	1200, 997, 835	2(2)				5(3)	
5a	GlcNAc–Gal–GlcNAc-6-OY	1302	1099, 937, 734					5(3)	
6	Gal–GlcNAc–Gal-3-OX	1362	1200, 997, 835	2(2)	3(2)			5(3)	
6a	GlcNAc–Gal–GlcNAc-6-OY	1302	1099, 937, 734					5(3)	
7	Fuc–Gal–$GlcNAc_2$-6-OY	1448	–	2(2)					
8	Fuc–Gal–GlcNAc–Gal-3-OX	1508	1362. 1200, 997, 835					5(3)	
9	Gal–GlcNAc–Gal–GlcNAc-3-OX	1565	1403, 1200, 1038, 835					5(3)	6(7)
10	Gal–GlcNAc–Gal–GlcNAc-6-OY	1464	1302, 1099, 937, 734		3(2)	3(3)		5(3)	6(7)
11	Fuc–Gal–GlcNAc–Gal–GlcNAc-3-OX	1711	1565, 1362, 1200, 997, 835	2(2)				5(3)	6(7)
11a	Fuc–Gal_2–$GlcNAc_2$-6-OY	1610	–	2(2)				5(3)	6(7)
12	GlcNAc–Gal–GlcNAc–Gal–GlcNAc-3-OX	1768	1565, 1403, 1200, 1038, 835						6(7)
13	Gal–GlcNAc–Gal–GlcNAc–Gal-3-OX	1727	1565, 1362, 835				4(3)		
14	Gal–GlcNAc \Gal-3-OX / Gal–GlcNAc	1727	1565, 1362, 1200, 937, 835		3(2)	3(3)			
15	Fuc–Gal_3–$GlcNAc_3$-OX	1874	–				5(3)		

[a]X = $CH[CH(NHAc)CH_2OH]CH_2$-lipid; Y = CH_2CH_2-lipid where lipid = $-NHCH_2CH_2OP(=O)(-OH)OCH_2CH[-OCO(CH_2)_{14}CH_3]CH_2OCO(CH_2)_{14}CH_3$.
[b]Mass values rounded down to the nearest nominal mass.

TABLE VII

The ^{1}H-n.m.r. chemical shifts (p.p.m. from d.s.s.) of the hexa- to hepta-saccharide fraction from meconium glycopeptides[a]

Residue	*Atom*	*J2(2),J3(2),J3(3)* 	*J4(3)* 	*J6(7)* 
GalNAcol	H-2	3.300, 4.505	4.406	4.289
	H-3	4.079	4.052	–
	H-4	3.455	–	–
	H-5	4.288, 4.272	4.192	4.140
	NAc	2.068, 2.067	2.048	2.034
GlcNAc^6	H-1	4.553		
	H-6	3.999		
	NAc	2.063, 2.059		
GlcNAc^3	H-1		4.692	4.649
	NAc		2.040	2.077
^{3}GlcNAc	H-1	4.721, 4.688	4.741	4.740
	NAc	2.028, 2.038	2.023	2.029
GlcNAc	H-1	4.608	4.705	4.558
	NAc	2.040	2.031	2.044
Gal^3	H-1	4.469, 4.464	4.464	
(to GalNAcol)	H-2	3.919	–	
	H-4	4.140	4.120	
Gal	H-1	4.470	4.453	4.472
	H-2	–	–	–
	H-4	3.919	4.146	4.152
Gal	H-1	4.443, 4.453	4.442	4.456
	H-2	–	3.525	

[a]For symbols, see Table II.

ity of a chain terminating β-Gal-(1→4)-GlcNAc as suggested by methylation analysis (Table III).

In fraction J4(3), a linear sequence, Hex–HexNAc–Hex–HexNAc–Hex was detected on t.l.c.–m.s. microsequencing of the neoglycolipid derivatives (band 13, Fig. 4) with $[M-H]^-$ at *m/z* 1727 and fragment ions with *m/z* 1565, 1362, 1200, 997, and 835. The absence of other major lipid bands indicated that this fraction consisted mainly of a single oligosaccharide without a 6-branch on the core GalNAcol. In accord with this inference was the fragment at *m/z* 1117 obtained on l.s.i.-m.s. of the methylated oligosaccharides representing Hex_3–$HexNAc_2$, and data from methylation analysis which indicated the presence of a hexasaccharide containing Gal-(1→), $[(\rightarrow 3)\text{-Gal}]_2$, $[(\rightarrow 3/4)\text{-GlcNAc}]_2$, and (→3)-GalNAcol. The ^{1}H-n.m.r. spectrum (Table VIII) was consistent with the presence of the following oligosaccharides with the internal Gal–GlcNAc sequence probably having a β-(1→3) linkage as described for an oligosaccharide of ovarian cyst mucins[10].

β-Gal-(1→4/3)-β-GlcNAc-(1→3)-β-Gal-(1→4/3)-β-GlcNAc-(1→3)-β-Gal-(1→3)-GalNAcol

Fractions J5(2) and J5(3) consisted of a complex mixture of hexa- and heptasaccharides as shown by l.s.i.–m.s. of the native and methylated oligosaccharides (Table V). Whereas ^{1}H-n.m.r. spectroscopy of this mixture was precluded, t.l.c.–m.s. microsequencing clearly indicated the 3 and 6 linkages to GalNAcol that are illustrated for J5(3) in Fig. 4 (see also Table VI). The results with J5(2) were similar (not shown), except for an additional band (band 15, Fig. 4) from J5(3) containing an extended 3-linked chain with an $[M-H]^-$ ion at *m/z* 1874 which was not detected in J5(2). The fragment ions from this band were of low intensity but consistent with the structure of a fucosylated heptasaccharide.

Fraction J6(7) was also complex, as seen from the l.s.i.-m.s. of native and methylated oligosaccharides (Table V). The t.l.c.–m.s. microsequencing procedure detected a major pentasaccharide component 3-linked to GalNAcol, with the linear sequence GlcNAc–Gal–GlcNAc–Gal–GlcNAc (band 12, Fig. 4). This sequence was also apparent from the fragment ion with *m/z* 1158 in the l.s.i.-mass spectrum of the methylated oligosaccharide fraction. Comparison of the ^{1}H-n.m.r. data (Table VII) with those in the literature suggested the presence of a component having a linear sequence with β-GlcNAc-(1→3)-GalNAcol forming the core as in trisaccharides already characterised[2,9,10]. The ^{1}H-n.m.r. data were also consistent with repeating β-Gal-(1→3)-GlcNAc sequences (Table VIII). However, methylation analysis of fraction J6(7) indicated an abundance of both →4)-GlcNAc and →3)-GlcNAc sequences and therefore only the following partial assignment can be proposed: β-GlcNAc-(1→3)-β-Gal-(1→3/4)-β-GlcNAc-(1→3)-β-Gal-(1→3/4)-β-GlcNAc-(1→3)-GalNAcol. Several fucosylated chains were also detected in J6(7) by t.l.c.–m.s. of the neoglycolipid products, including Fuc–Gal–GlcNAc (band 3b, Fig. 4) and Fuc–Gal–GlcNAc–Gal–GlcNAc (band 11) 3-linked to GalNAcol, and Fuc–Gal_2–$GlcNAc_2$ (band 11a) 6-linked to GalNAcol. From the compositions of oligosaccharides present in this fraction (Table V), these bands were considered to arise from several 3,6-branched structures.

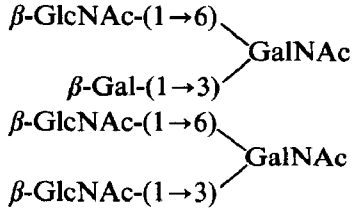
DISCUSSION

In this study, the resolving power and sensitivity of the t.l.c.–m.s. microsequencing procedure[5] has been exploited, whereby mixtures of oligosaccharide-alditols were first subjected to controlled periodate oxidation, which cleaved the C-4–C-5 bond of core GalNAcol, and the resulting fragments were conjugated to phosphatidylethanolamine dipalmitoate. The products gave diagnostic ions in l.s.i.-m.s. which permitted assignment of sequences that were 3- and 6-linked to the core monosaccharide. This procedure, in conjunction with m.s. and ^{1}H-n.m.r. spectroscopy of the mixtures of oligosaccharides, has been used to charactise sequences not identified previously in two highly heterogeneous fractions of human meconium and has extended the data base for O-linked oligosaccharides from this source.

From the present results, together with those from earlier studies[2–5] and those of fucosylated, sialylated, and sulphated sequences[14], the structures of the oligosaccharides of human meconium glycoproteins can be summarised as follows.

(*a*) Sequences in the core region:

GalNAc
β-Gal-(1→3)-GalNAc
β-GlcNAc-(1→3)-GalNAc
β-GlcNAc-(1→6)-GalNAc
α-GalNAc-(1→3)-GalNAc



(*b*) Backbone regions consisting of alternating Gal and GlcNAc residues joined to the core GalNAcol either via 3-linked Gal:

β-Gal-(1→3)
β-Gal-(1→3)-β-GlcNAc-(1→3)-β-Gal-(1→3)
β-Gal-(1→4)-β-GlcNAc-(1→3)-β-Gal-(1→3)
β-Gal-(1→4)-β-GlcNAc-(1→6)-β-Gal-(1→3)
β-Gal-(1→?)-β-GlcNAc-(1→3)-β-Gal-(1→?)-β-GlcNac-(1→3)-β-Gal-(1→3)
β-Gal-(1→?)-β-GlcNAc-(1→6)
β-Gal-(1→3)
β-Gal-(1→?)-β-GlcNAc-(1→3)

or 3-linked GlcNAc:

β-GlcNAc-(1→3)
β-Gal-(1→3)-β-GlcNAc-(1→3)
β-Gal-(1→4)-β-GlcNAc-(1→3)
β-GlcNAc-(1→3)-β-Gal-(1→?)-β-GlcNAc-(1→3)
β-Gal-(1→3)-β-GlcNAc-(1→3)-β-Gal-(1→3)-β-GlcNAc-(1→3)
β-Gal-(1→3)-β-GlcNAc-(1→3)-β-Gal-(1→4)-β-GlcNAc-(1→3)
β-GlcNAc-(1→3)-β-Gal-(1→?)-β-GlcNAc-(1→3)-β-Gal-(1→?)-β-GlcNAc-(1→3)

or 6-linked GlcNAc:

β-GlcNAc-(1→6)
β-Gal-(1→4)-β-GlcNAc-(1→6)
β-Gal-(1→3)-β-GlcNAc-(1→3)-β-Gal-(1→4)-β-GlcNAc-(1→6)

The linkages designated ? have not been substantiated as either 3 or 4 linkages, or as a mixture of 3 and 4 linkages. However, from the assignments which could be made, it is clear that both the type 1 [β-Gal-(1→3)-β-GlcNAc-(1→] and type 2 [β-Gal-(1→4)-β-GlcNAc-(1→] sequences can occur on branches that are 3- or 6-linked to core GalNAcol, although, so far, only the type 2 sequence 6-linked to the core has been found. Of the structures elucidated in the present study, the sequences β-Gal-(1→3/4)-β-GlcNAc-(1→3)-β-Gal-(1→3/4)-β-GlcNAc-(1→3)- joined to the β-Gal-(1→3)-GalNAcol core have not been identified previously in meconium, although the sequence with two internal →3)-β-GlcNAc-(1→ residues has been described in ovarian cyst mucin[10]. Similarily, the disaccharide β-Gal-(1→3)-β-GlcNAc-(1→ and β-Gal-(1→4)-β-GlcNAc-(1→ chains linked to →3)-β-Gal-(1→3)-GalNAcol have not been seen in meconium, although they are commonly found in other human mucins[9–11]. A second extended backbone, β-GlcNAc-(1→3)-β-Gal-(1→3/4)-β-GlcNAc-(1→3)-β-Gal-(1→ joined to →3/4)-β-GlcNAc-(1→3)-GalNAcol, and a shorter sequence having terminal β-GlcNAc-(1→ are uncommon.

(*c*) The terminal Gal residues of meconium oligosaccharides may be substituted with α-Fuc-(1→2)-, α-GlcNAc-(1→4)-, α-GalNAc-(1→3)-, α-NeuAc-(2→3)-, or HSO_3-3; the subterminal GlcNAc residue may be substituted[14] with α-Fuc-(1→3)- and the core GalNAc residue with α-NeuAc-(2→6)-. It is of interest whether the α-GalNAc-(1→3)-β-Gal-(1→3/4)-β-GlcNAc-(1→3)-GalNAcol oligosaccharide found in meconium, from presumed blood group O neonates, arises from a blood group A sequence after defucosylation or represents blood group A-like antigenic activity as discussed[3]. The terminal β-GlcNAc-(1→3)-Gal sequence probably represents an intermediate in the biosynthesis which is normally capped by a Gal residue.

The occurrence in meconium glycoproteins of a greater diversity of oligosaccharide sequences than in human bronchial mucins or ovarian cyst glycoproteins, for example, is likely to reflect the mixed origin of meconium. This origin probably includes glycopro-

teins of the amniotic fluid and cells therein, and gastrointestinal and possibly bronchial secretions of the neonate.

REFERENCES

1 T. Feizi, *Nature (London)*, 314 (1985) 53–57.
2 E. F. Hounsell, A. M. Lawson, J. Feeney, H. C. Gooi, N. J. Pickering, M. S. Stoll, S. C. Lui, and T. Feizi, *Eur. J. Biochem.*, 148 (1985) 367–377.
3 E. F. Hounsell, A. M. Lawson, J. Feeney, G. C. Cashmore, D. P. Kane, M. Stoll, and T. Feizi, *Biochem. J.*, 256 (1988) 397–401.
4 E. F. Hounsell, A. M. Lawson, M. S. Stoll, D. P. Kane, G. C. Cashmore, R. A. Carruthers, J. Feeney, and T. Feizi, *Eur. J. Biochem.*, 186 (1989) 567–610.
5 M. S. Stoll, E. F. Hounsell, A. M. Lawson, W. Chai, and T. Feizi, *Eur. J. Biochem.*, 189 (1990) 499–507.
6 I. Ciucanu and F. Kerek, *Carbohydr. Res.*, 131 (1984) 209–217.
7 A. M. Lawson, W. Chai, G. C. Cashmore, M. S. Stoll, E. F. Hounsell, and T. Feizi, *Carbohydr. Res.*, 200 (1990) 47–57.
8 E. F. Hounsell and D. J. Wright, *Carbohydr. Res.*, 205 (1990) 19–29.
9 G. Lamblin, A. Boersma, M. Lhermitte, P. Roussel, J. H. G. M. Mutsaers, H. van Halbeek, and J. F. G. Vliegenthart, *Eur. J. Biochem.*, 143 (1984) 227–236.
10 J. H. G. M. Mutsaers, H. van Halbeek, J. F. G. Vliegenthart, A. M. Wu, and E. A. Kabat, *Eur. J. Biochem.*, 157 (1986) 139–146.
11 J. Breg, H. van Halbeek, J. F. G. Vliegenthart, G. Lamblin, M.-C. Houvenaghel, and P. Rousell, *Eur. J. Biochem.*, 168 (1987) 57–68.
12 B. N. Rao, V. K. Dua, and C. A. Bush, *Biopolymers*, 24 (1985) 2207–2229.
13 J. Breg, H. van Halbeek, J. F. G. Vliegenthart, A. Klein, G. Lamblin, and P. Roussel, *Eur. J. Biochem.*, 171 (1988) 643–654.
14 C. Capon, Y. Leroy, J.-M. Wieruszeski, G. Ricart, G. Strecker, J. Montreuil, and B. Fournet, *Eur. J. Biochem.*, 182 (1989) 139–152.