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OLIGOSACCHARIDES FROM FAECES OF A BLOOD-GROUP B, BREAST-FED INFANT*

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ABSTRACT

Eight oligosaccharides have been isolated from faeces of a blood group B, secretor, breast-fed infant and characterized by sugar and methylation analysis, f.a.b. mass spectrometry and ^{1}H-n.m.r. spectroscopy. One of these oligosaccharides has not previously been reported and is a tri-L-fucosyl derivative of lacto-*N*-hexaose. The other compounds were identical to oligosaccharides found in human milk. Several of the reported compounds require the secretor dependent 2′-fucosyltransferase for their biosynthesis. Since the mother of this child was an O(H) non-secretor, an intestinal biosynthesis of at least some of these compounds is strongly indicated. No blood group B active oligosaccharides were detected which is in sharp contrast to the oligosaccharide excretion in faeces from a blood group A infant [Sabharwal *et al.*, *Mol. Immunol.*, 21 (1984) 1105–1112] in which all the major oligosaccharides isolated were blood group A active.

INTRODUCTION

Human milk is a rich source of oligosaccharides[1,2]. Some of these oligosaccharides are synthesized from about the 13th week of pregnancy and are excreted in urine during both pregnancy and lactation[3].

The secretor and Lewis phenotype of the woman determine which structures can be found in milk and urine. Blood group H-active structures, for instance, are

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only present in milk or urine of secretors, and the Lewis gene is a prerequisite for the formation of Lewis-active oligosaccharides. Blood group A and B active oligosaccharides have not been found in milk, but are characteristic components in urine of ABH-secretors[4], the amount of which can be enhanced considerably by ingestion of D-galactose or lactose[5]. Experiments have indicated an intestinal origin of these oligosaccharides[6].

In order to study the intestinal survival of milk oligosaccharides and also to look for a possible biosynthetic activity in the intestine, faeces from newborn, breast-fed infants have been analysed for oligosaccharide content. Four blood group A-active oligosaccharides have been isolated from faeces of a blood group A, secretor infant[7]. These oligosaccharides are most probably formed in the intestine with the milk oligosaccharides acting as precursors in their biosynthesis. The oligosaccharide composition of faeces from a blood group B breast-fed infant is now reported.

EXPERIMENTAL

Materials. — Faeces produced during 48 h were collected from a 15-week-old, healthy, breast-fed, blood group B, Le^{a-b+}, secretor infant. Samples were kept frozen at −20° until analysed. The mother of this child was an O(H) non-secretor and had the Le^{a+b-} phenotype.

The faeces were suspended in water to which phenylmercuric nitrate (30 mL of a saturated solution in water) was added to prevent bacterial growth. The extract was filtered through glass wool and then ultrafiltered through an Amicon PM 30 Diaflo^{R} ultrafiltration membrane (Amicon Corp., Lexington, MA, U.S.A.). The ultrafiltrate was then lyophilised.

The dry ultrafiltrate (5.75 g) was dissolved in 0.02M pyridine–acetic acid buffer (pH 5.4; 45 mL). A portion of the solution (15 mL) was applied to a Sephadex G-15 (Pharmacia, Sweden) column (144 × 5 cm) which was eluted with the same buffer.

Methods. — ^{1}H-N.m.r. spectra were recorded with a Bruker AM-500 F.t.-spectrometer. The oligosaccharides were treated with D_2O, lyophilised, and dissolved in high purity D_2O (99.95%, Ciba–Geigy, Switzerland). The chemical shifts are expressed relative to the signal of internal sodium 4,4-dimethyl-4-silapentanesulfonate (DSS), but were actually measured relative to the signal of internal acetone (δ 2.225 downfield from the signal of DSS). The spectra were recorded at 25°. Fast-atom-bombardment (f.a.b.) positive-ion mass spectra were recorded with a VG Analytical ZAB-SE mass spectrometer. The primary beam was composed of Xe atoms with an energy of 8 keV. The ions were accelerated by a potential of 10 kV, the samples were dissolved in thioglycerol, and the spectra were processed with an on-line VG Analytical 11-250 system DEC PDP-11/24 computer.

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chromatography in the following solvent systems: (*A*) 3:1:1 (v/v) ethyl acetate–acetic acid–water; (*B*) 5:5:1:3 (v/v) ethyl acetate–pyridine–acetic acid–water; (*C*) 2:1:2 (v/v) ethyl acetate–pyridine–water, upper phase; and (*D*) 6:1:3 (v/v) propanol–ethyl acetate–water. Paper chromatograms were stained with a silver dip reagent[8]. A column (90 × 1.6 cm) packed with Zerolit SRC-10 (K^+) cation-exchange resin (Diamond Shamrock, Isleworth, U.K.) was used for recycling chromatography[9]. The high-pressure liquid chromatography (l.c.) apparatus consisted of the following equipment (Waters Associates, Milford MA, U.S.A.): a solvent delivery module (model 590), a universal injector (model U6K), a refractive index detector (model 401), and a variable wavelength u.v. detector (model 481 Lambda-Max TM). Nucleosil^R C-18 semi-preparative reverse-phase columns (300 × 10 mm) were used for all the studies. Distilled and de-ionized water was used as eluent, unless otherwise stated. The oligosaccharides containing acetamido groups were detected by absorbance at 206 nm. In all experiments, the flow rate was maintained at 3 mL/min. The l.c. separations were basically as previously described[10,11].

Colourimetric methods were used for the determination of total hexose[12]. Sugar and methylation analyses were performed[13], and permethylated oligosaccharide alditols were prepared as previously described[14].

RESULTS

The crude mixture of ultrafiltered faeces extract was fractionated on a

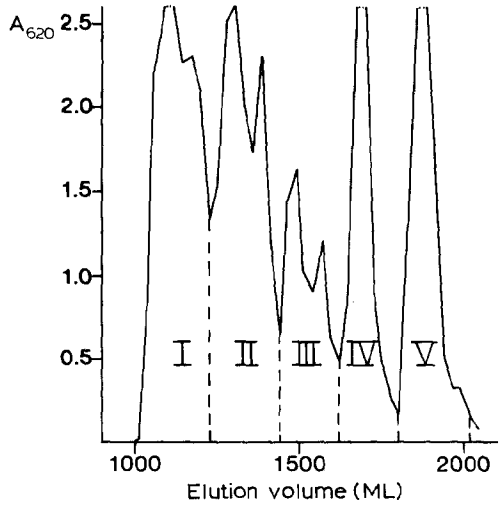


Fig. 1. Gel chromatographic profile of ultrafiltered and concentrated faeces on a Sephadex G-15 column. Eluted fractions were analysed for total hexose and pooled into fractions I–V as indicated.

TABLE I

STRUCTURES AND AMOUNTS OF COMPOUNDS ISOLATED FROM FAECES OF THE BLOOD GROUP B, SECRETOR, BREAST-FED INFANT

Fraction	*Trivial name*	*Structure*	*Amount in 24-h portion of faeces (mg)*
I_1	Trifucosylated lacto-*N*-hexaose	**1**	9
II_1	Lacto-*N*-difucohexaose I	**2**	43
II_2	Lacto-*N*-fucopentaose III	**3**	5
II_3	Lacto-*N*-fucopentaose II	**4**	5
II_4	Lacto-*N*-fucopentaose I	**5**	8
II_5	Lacto-*N*-tetraose	**6**	28
III_1	Lactodifucotetraose	**7**	11
III_2	3-Fucosyllactose	**8**	8

TABLE II

SUGAR COMPOSITION OF COMPOUNDS **1–8** ISOLATED FROM FRACTIONS I–III

Fractions	*Compounds*	*Molar proportions relative to Glc*			
		Fuc	*Gal*	*Glc*	*GlcNAc*
I_1	**1**	2.9	2.6	1.0	2.5
II_1	**2**	1.6	1.8	1.0	0.9
II_2	**3**	1.0	2.0	1.0	1.0
II_3	**4**	1.0	2.0	1.0	1.0
II_4	**5**	0.6	1.6	1.0	1.0
II_5	**6**	0	1.6	1.0	1.1
III_1	**7**	1.8	1.0	1.0	0
III_2	**8**	0.6	0.9	1.0	0

TABLE III

METHYLATION DATA OF COMPOUNDS **1–8** ISOLATED FROM FRACTIONS I–III

Sugar	*Fractions*[a]							
	I_1	*II_1*	*II_2*	*II_3*	*II_4*	*II_5*	*III_1*	*III_2*
1,2,3,5,6-Me-Glc	0.7	0.4	0.3	0.2	0.6	0.4	0	0
1,2,5,6-Me-Glc	0	0	0	0	0	0	0.6	0.3
2,3,4-Me-Fuc	2.5	1.3	0.5	0.5	1.3	0	1.5	0.7
2,3,4,6-Me-Gal	0.8	0	0.5	0.6	0	0.8	0	1.0
2,4,6-Me-Gal	0	1.0	1.0	1.0	1.0	1.0	0	0
3,4,6-Me-Gal	1.4	0.7	0	0	1.7	0	1.0	0
2,4-Me-Gal	1.0	0	0	0	0	0	0	0
4,6-Me-GlcN(Me)Ac	0	0	0	0	[b]	[b]	0	0
6-Me-GlcN(Me)Ac	[b]	[b]	[b]	[b]	0	0	0	0

[a]Relative molar proportions calculated by setting the appropriate sugar derivative to 1.0. [b]Present but not quantitatively determined.

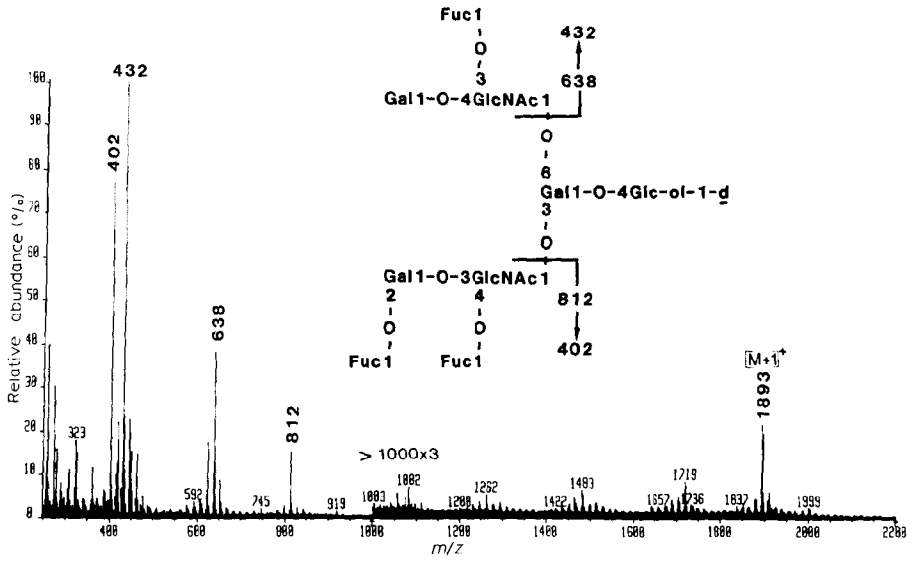
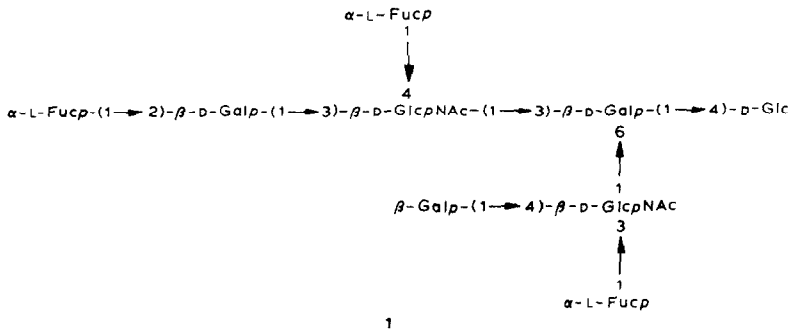


Fig. 2. F.a.b.–m.s. of **1** as a permethylated oligosaccharide alditol.

Sephadex G-15 column. Eluted fractions (8 mL) were assayed for total hexose and pooled into Fractions I–V (Fig. 1).

Fraction I. — The material from Fraction I was analysed by l.c. in 8% methanol in water as the eluent. The majority of the sugars was rapidly eluted from the column and this material was rechromatographed and eluted with water. One major and several minor components were obtained. However, the latter were in too low amounts to be characterized. The major component was rechromatographed several times until apparently pure (Fraction I_1, Table I). This fraction was subjected to structural analysis. Sugar analysis of the reduced ($NaBD_4$) compound as alditol acetates showed Fuc, Gal, Glc, and GlcNAc in the proportions 2.9:2.6:1.0:2.5, respectively (Table II). When analysed by g.l.c.–m.s., complete and exclusive incorporation of deuterium was seen in the Glc residue, indicating that Glc was the only reducing terminal residue and that Glc residues did not occur anywhere else in the molecule. The methylation analysis showed nonreducing terminal groups of Fuc and Gal and the reducing terminal Glc residue to be 4-*O*-substituted (Table III). The internal sugars were 2-mono- and 3,6-di-*O*-substituted Gal residues, as well as a 3,4-di-*O*-substituted GlcNAc residue.

Additional structural information was obtained by f.a.b.–m.s. of the permethylated oligosaccharide alditol (Fig. 2). Information about the substitution pattern of the di-*O*-substituted GlcNAc residues was obtained from the primary fragments at *m/z* 638 and 812, and the secondary fragments formed by eliminations from these ions. The fragment at *m/z* 638 showed that the GlcNAc residue is substituted by one Fuc and one Gal residue. The fragment at *m/z* 812 showed an additional Fuc residue. The methylation analysis showed a 3,4-di-*O*-substituted



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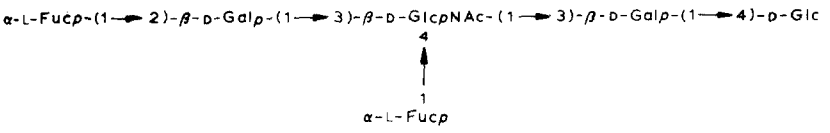

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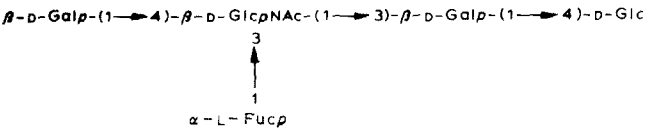

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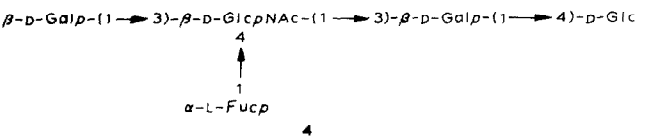

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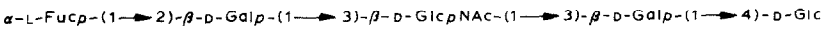

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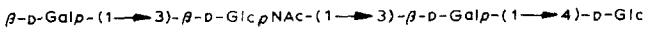

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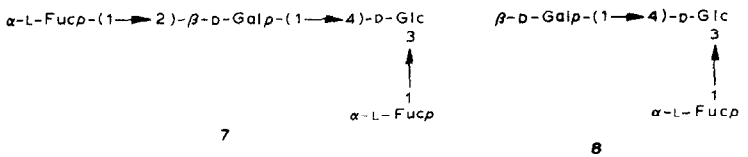

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GlcNAc residue as the only hexosamine derivative (Table III). The positions of substitution of the GlcNAc residues were determined from the secondary fragments at *m/z* 402 and 432 formed by eliminations from fragments at *m/z* 638 and 812, respectively. For a substituted hexosamine residue, the elimination occurred

TABLE IV

PARTIAL ASSIGNMENT OF ^{1}H-N.M.R. SPECTRA OF COMPOUNDS ISOLATED FROM FRACTIONS I–III

Residue	*Atom*	*Fraction*							
		I_1	II_1	II_2	II_3	II_4	II_5	III_1	III_2
α-D-Glc	H-1	5.217	5.218	5.219	5.218	5.218	5.219	5.174	5.181
β-D-Glc	H-1	4.663	4.661	4.661	4.662	4.660	4.661	4.621	4.652
β-D-Glc	H-2	3.289	3.277	3.278	3.278	3.278	3.278		
β-D-Gal*p*-(1→4)	H-1	4.409	4.420	4.434	4.432	4.418	4.441	4.490	4.432
	H-4	4.117	4.135	4.156	4.154	4.136	4.152		
β-D-Gal[a]	H-1	4.654	4.660		4.504	4.645	4.441		
β-D-Gal[b]	H-1	4.450		4.461					
β-D-Glc*p*NAc[a]	H-1	4.600	4.603		4.693	4.621	4.729		
	H-3	4.127	4.130		4.079				
	CH_3	2.051	2.062		2.032	2.056	2.028		
β-D-Glc*p*NAc[b]	H-1	4.642		4.713					
	CH_3	2.058		2.022					
α-L-Fuc*p*-(1→2)	H-1	5.149	5.150			5.186		5.279	
	H-5	4.342	4.342			4.290		4.293/4.253[c]	
	H-6	1.273	1.274			1.234		1.264	
α-L-Fuc*p*-(1→3)	H-1	5.101		5.127				5.393/5.447[c]	5.382/5.439[c]
	H-5	4.822		4.838				4.874/4.860[c]	4.835/4.822[c]
	H-6	1.175		1.176				1.243	1.187
α-L-Fuc*p*-(1→4)	H-1	5.025	5.026		5.028				
	H-5	4.860	4.870		4.877				
	H-6	1.259	1.259		1.181				

[a]Residue in type I chain. [b]Residue in type II chain. [c]α and β anomer of Glc residue.

compounds with known structures, such as lacto-*N*-fucopentaose II (**4**), lacto-*N*-fucopentaose III (**3**), and lacto-*N*-difucohexaose I (**2**)[15]. The fragment at *m/z* 638 gave a secondary fragment of *m/z* 432 which showed that the Fuc residue is linked to O-3, and consequently the Gal residue to O-4 of the GlcNAc residue. The corresponding secondary fragment from that at *m/z* 812 was at *m/z* 402 indicating that a Fuc-Gal sequence is linked to O-3, and that a Fuc residue must be linked to O-4 of the GlcNAc residue. The ion at *m/z* 1893 is the molecular ion plus one mass unit, which is consistent with nonasaccharide **1**. The methylation analysis showed a branched Gal residue substituted at O-3 and O-6. The branches were Gal-(1→4)-[Fuc-(1→3)]-GlcNAc-(1→ and Fuc-(1→2)-Gal-(1→3)-[Fuc-(1→4)]-GlcNAc-(1→, but the mass spectrum gave no information concerning which branch is attached to O-3 and O-6 of this Gal residue.

The compound was further analysed by ^{1}H-n.m.r. spectroscopy. Assignments were done with 2D correlation spectroscopy[16] (COSY) and comparison with lacto-*N*-fucopentaose I (**5**), lacto-*N*-fucopentaose II (**4**), lacto-*N*-fucopentaose III (**3**), and lacto-*N*-difucohexaose I (**2**). The spectrum of Fraction I_1 is shown in Fig. 3(B) together with a spectrum of **2** (A). The anomeric region showed a nonasaccharide having a Glc residue substituted at O-4 at the reducing end [δ 5.217 (H-1α), 4.663 (H-1β), and 3.289 (H-2β)]. The doublet at δ 4.117 was assigned to H-4 of a β-D-Gal

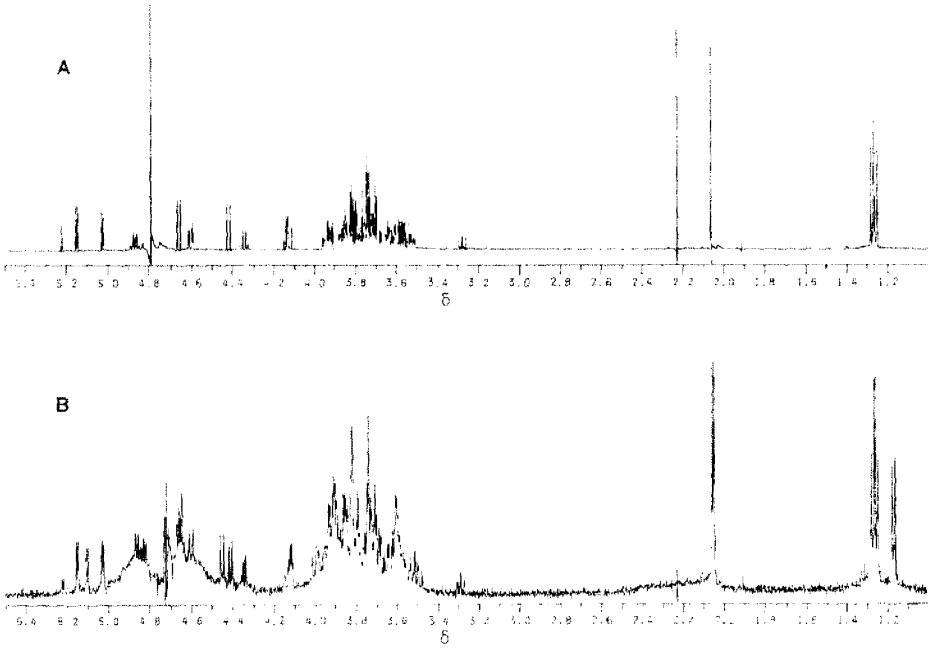

Fig. 3. 500-MHz ^{1}H-n.m.r. spectrum for a solution in D_2O at 25° of: (A) lacto-*N*-difucohexaose I (**2**); (B) compound **1**.

preferentially from O-3. This was confirmed by comparing spectra of model residue which is substituted at O-3. The COSY-spectrum connected this resonance with the signal of the β-D anomer at δ 4.409. The β-D anomer of the Gal residue substituted at O-2 gave a signal shifted downfield to δ 4.654, and the terminal Gal group showed a β-D anomer signal at δ 4.450. Comparing the pattern for the α-L-Fuc group of compound I_1 with those of **4**, **3**, and **2**, the following assignments could be made; for α-L-Fuc*p*-(1→2), H-1 at δ 5.149, H-5 at δ 4.342, and H-6 at δ 1.273; for α-L-Fuc*p*-(1→3), H-1 at δ 5.101, H-5 at δ 4.822, and H-6 at δ 1.175; and for α-L-Fuc-(1→4), H-1 at δ 5.025, H-5 at δ 4.860, and H-6 at δ 1.259 (Table IV). The signals for H_2-6 of α-L-Fuc*p*-(1→4) and -(1→2) displayed the same patterns as those for **2** (see Fig. 3). The signals from the GlcNAc residue linked to O-3 of the branched Gal residue showed the same pattern as that of **2**, *i.e.*, H-1β at δ 4.600, H-2 at δ 3.851 and a typical downfield shift of the H-3 signal at δ 4.127. The signal for H-1β of the GlcNAc residue linked to O-6 of the branched Gal residue was found at δ 4.642. Its doublet pattern was obscured owing to strong coupling between H-2 and H-3, the signals of which were found at δ ~3.90. The ^{1}H-n.m.r. spectrum confirmed the structural information obtained from the mass spectrum, indicating the anomeric configuration of the sugar residues, and furthermore showing that the difucosylated branch is linked to O-3 of the branched Gal residue.

Fraction II. — Fraction II from the Sephadex G-15 column (Fig. 1) was further fractionated, by recycling chromatography and l.c., into Fractions II_1, II_4,

partially separated, and were purified by preparative paper chromatography in system *C* for three days. The purity of all components (II_1–II_5) was finally monitored by analytical paper chromatography in systems *A*, *B*, and *D*. All five components were then subjected to sugar and methylation analyses, and the results (Tables II and III) suggest that they are common milk oligosaccharides. The components were also studied by ^{1}H-n.m.r. spectroscopy to confirm their structures. The chemical shifts are given in Table IV.

Fraction III. — Fraction III from the Sephadex G-15 column (Fig. 1) was further fractionated by recycling chromatography and l.c., to give Fraction III_1 in pure form. Fraction III_2, however, was further purified by preparative paper chromatography. Chemical analyses and ^{1}H-n.m.r. data indicated structures **7** and **8**.

Fractions IV and V. — These fractions were subjected to analytical paper chromatography. Many mono- and di-saccharides were observed which were not studied further. Only trace amounts of lactose were observed.

DISCUSSION

The complete structure of complex carbohydrates may frequently be deduced by a combination of sugar analysis, methylation analysis, f.a.b.–m.s. and n.m.r. spectroscopy. This is illustrated by the elucidation of the structure of Fraction I_1 (compound **1**). From the sugar and methylation analyses, a number of possible isomeric structures could be constructed, although no information on the anomeric configurations was obtained. Mass spectrometry, in particular f.a.b.–m.s., gave a lot of important information, especially regarding the substitutions by L-fucosyl groups, but no data on anomeric configuration. In the case of **1**, it was not possible to differentiate between two isomers resulting from branching at the Gal residue attached to the terminal reducing Glc residue. The aforementioned problems could be solved by comparison of the ^{1}H-n.m.r. spectrum with those of reference compounds. The difucosylated branch (Type I chain) is linked to O-3 and the monofucosylated branch (Type II chain) to O-6 of the Gal residue. The non-fucosylated core structure of **1** is identical with that of the known milk oligosaccharide, lacto-*N*-hexaose.

Human milk is the most likely origin of the oligosaccharides reported herein, as formula-fed children have no oligosaccharides of this type in their faeces[17]. The mother of this B-secretor child had the Le^{a+b-} phenotype and, thus, her milk lacks oligosaccharides with a L-fucosyl group linked α-(1→2) to a D-galactose residue. The initial impression obtained is that the oligosaccharides isolated from faeces are merely oligosaccharides from the ingested milk which have survived passage through the alimentary canal. The major milk oligosaccharide, lactose, is completely degraded, but the higher mol. wt. oligosaccharides, being resistant to lactase degradation, remain. The striking observation is the presence of oligosaccharides containing α-L-Fuc*p*-(1→2)-D-Gal groups, which are absent from the mother's milk

since she is a non-secretor of H-substances. This finding strongly indicates an intestinal biosynthetic activity, as the infant is a secretor of H-substances and is, therefore, capable of biosynthesising these structures. It may also be suggested that this biosynthetic process takes place at the distal end of the small intestine where the ingested lactose would already have been degraded into galactose and glucose, since a more proximal site would have led to production of some 2′-fucosyllactose from the great excess of lactose still present. Any 2′-fucosyllactose formed would be expected to survive lactase attack and appear in the faeces.

These results give a further example of glycosylation of oligosaccharides in the intestine. In an earlier study[7], we showed that faeces from a blood group A infant contained A-active oligosaccharides resulting from a modification of milk oligosaccharides. No corresponding B-active oligosaccharides, however, were found in the infant studied here. This could be due to the biosynthesis, by this infant, of B-active structures, requiring two biosynthetic steps since the mother is a non-secretor. Thus, insufficient amounts of α-L-Fuc*p*-(1→2)-D-Gal-containing oligosaccharides are available to act as substrate for the *B*-enzyme. Additionally, the *B*-enzyme is known to have a higher K_m than the *A*-enzyme and, thus, requires a higher substrate concentration in order to act effectively. Analysis of faeces from other infants will clarify these points.

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