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SEPARATION OF HUMAN MILK OLIGOSACCHARIDES BY RECYCLING CHROMATOGRAPHY. FIRST ISOLATION OF LACTO-*N*-*neo*-DIFUCOHEXAOSE II AND 3′-GALACTOSYLLACTOSE FROM THIS SOURCE*

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ABSTRACT

Lacto-*N*-*neo*-difucohexaose II, β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-D-Glc, and 3′-galactosyllactose, β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc, were isolated for the first time from human milk by means of a recycling chromatography technique. Through this method, carried out mainly on columns of K^+ ion-exchange resins and either Bio-Gel P-4 or TSK 40W(S) gel filtration media, up to one gram of an oligosaccharide mixture could be handled and lacto-*N*-*neo*-difucohexaose II separated from isomeric lacto-*N*-difucohexaose I, α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc, and II, β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-D-Glc. This method also permitted resolution of isomeric mixtures of the trisaccharides 2′-fucosyllactose, α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-D-Glc, and 3-fucosyllactose, β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-D-Glc, the tetrasaccharides lacto-*N*-tetraose, β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc, and lacto-*N*-*neo*-tetraose, β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc, and the pentaoses lacto-*N*-fucopentaose I, α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc, II, β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc, and III, β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc, which have proved difficult if not impossible to separate by other means. The isolation of these and other milk oligosaccharides is described herein. The 500-MHz ^{1}H-n.m.r. spectra of lacto-*N*-*neo*-difucohexaose II and 3′-galactosyllactose, and their alditols, are recorded. ^{1}H-N.m.r. data on some other milk oligosaccharides, both natural and reduced, are also given.

*Dedicated to Professor Walter T. J. Morgan.

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1	Lactose	β-D-Gal*p*-(1→4)-D-Glc
2	2′-Fucosyllactose	α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-D-Glc
3	3-Fucosyllactose	β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-D-Glc
4	3′-Galactosyllactose	β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glc
5	6′-Galactosyllactose	β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-D-Glc
6	Lacto-*N*-tetraose	β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc
7	Lacto-*N*-*neo*-tetraose	β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc
8	Lactodifucotetraose	α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-D-Glc
9	Lacto-*N*-fucopentaose I	α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc
10	Lacto-*N*-fucopentaose II	β-D-Gal*p*-(1→3)-[α-L-Fuc*p*-(1→4)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc
11	Lacto-*N*-fucopentaose III	β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc
12	Lacto-*N*-difucohexaose I	α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc 4 ↑ 1 α-L-Fuc*p*
13	Lacto-*N*-difucohexaose II	β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc 4 3 ↑ ↑ 1 1 α-L-Fuc*p* α-L-Fuc*p* *4* *3* *2* *1*
14	Lacto-*N*-*neo*-difucohexaose II	β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc 3 3 ↑ ↑ 1 1 α-L-Fuc*p* α-L-Fuc*p* *4′* *2′*

Scheme 1. Structures and trivial names of milk oligosaccharides.

INTRODUCTION

The oligosaccharides first isolated from human milk by Kuhn *et al.*[1] have been of the utmost value to the field of glycoconjugate research. Interest in these compounds arose because of their growth factor properties[2] for *Lactobacillus bifidus Penn.*, but their application to other fields was soon demonstrated when Morgan and Watkins used them in inhibition studies to deduce the structures of the Le^a and Le^b blood group determinants[3]. The number of identified oligosaccharides has since increased[4–6], and many structural homologies have been recognised between these compounds and carbohydrates carried on glycoproteins and glycolipids. The milk oligosaccharides have been of use as substrates in the study of glycosidases and glycosyltransferases, as model structures during the development of many of the analytical techniques and structural methods used in studies of glycoproteins and glycolipids, and for determining the structural requirements of monoclonal antibodies (see refs. 7–10 for examples of these applications).

The isolation of these oligosaccharides has usually made considerable use of paper chromatography which is very time consuming when large quantities of sugars need to be separated, and this technique is incapable of resolving some of the isomeric mixtures involved, for instance that of the pentaoses **10** and **11**. Recycling chromatography[11–13] on columns of K^+ cation-exchange resins[14] has previously been shown[15] to resolve mixtures of both **2** and **3**, and of **6** and **7**. The extension of this technique to the resolution of mixtures of pentaoses and hexaoses is reported herein, which demonstrates that recycling chromatography on both K^+ ion-exchange resins and conventional gel filtration media, such as Bio-Gel P-4 and TSK Fractogel, is capable of separating complex mixtures of isomers.

EXPERIMENTAL

500-MHz ^{1}H-n.m.r. spectroscopy. — Solutions in D_2O of the samples were repeatedly evaporated, and the spectra were recorded, for solutions in D_2O, at 295 K as described earlier[16]. Chemical shifts (δ) were measured (in p.p.m.) from the signal of an acetone internal standard (δ 2.225) and expressed from the signal of sodium 4,4-dimethyl-4-silapentane-1-sulphonate.

Chromatography. — Silica gel 60 HPTLC plates were run in 6:5:4 (v/v) butanol–acetone–water. Descending paper chromatography was carried out on Whatman No. 40 paper eluted with 2:1:1 (v/v) (solvent *A*) and 10:4:3 (v/v) (solvent *B*) ethyl acetate–pyridine–water.

Analyses. — Neutral sugars and hexosamines were determined as described previously[17].

Materials. — The ion-exchange resin Zerolit 225-X4 (H^+; −200 mesh) was obtained from Diamond Shamrock Co. Ltd. Isleworth (U.K.), and fines were removed by repeatedly allowing the resin to settle in water over 40 min. It was then converted to the K^+ form with KOH. Bio-Gel P-4 (−400 mesh), AG 1-X4 (AcO^-;

200–400 mesh), and AG 50W-X8 (H^+; 200–400 mesh) ion-exchange resins were from Bio-Rad Laboratories, Watford (U.K.). Fractogel TSK HW-40(S), Silica Gel 60 HPTLC plates, and charcoal were purchased from BDH Ltd., Poole (U.K.). The Bio-Gel and Fractogel media were de-fined before use as described for the Zerolit resin. The other chemicals used were of analytical grade when obtainable.

Reduction. — Oligosaccharides were reduced with a 10-fold excess of $NaBH_4$, the excess borohydride was destroyed with acetic acid, Na^+ ions were adsorbed on AG 50W(H^+) cation-exchange resin, and boric acid was removed by repeated additions and evaporations of methanol.

Methylation. — Oligosaccharides were methylated with methyl iodide in *N,N*-dimethylformamide with BaO–$Ba(OH)_2$ as catalyst, and the methylated derivatives were hydrolysed and the methyl ethers identified as already described[17].

The recycling apparatus. — A schematic diagram of the arrangement used is shown in Fig. 1. The apparatus consists of two three-way valves, V_1 and V_2 (Locarte Co. London, U.K.), a twin-channel peristaltic pump (LKB Ltd., Croydon, U.K.), columns fitted with adjustable plungers (Amicon, Stonehouse, Glos, U.K. or Pharmacia Ltd., Milton Keynes, U.K.), a Knauer differential refractometer (Roth Scientific, Farnborough, U.K.), and a Philips PM8251 chart recorder. The tubing was of 0.5-mm P.T.F.E. joined by 14/28 flange connectors.

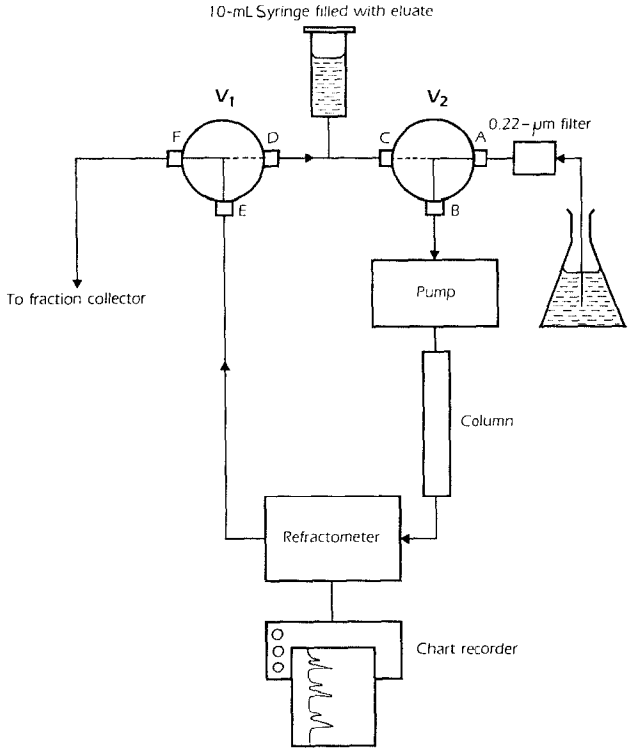

Fig. 1. Schematic diagram of the recycling apparatus.

During sample loading, ports D and C of valves V_1 and V_2 were closed, and the sample and following eluent were drawn in by the pump and forced through the column and refractometer, and out of the circuit *via* ports E and F of V_1. Typically, the column was eluted in this way until the region of interest reached the refractometer. Then ports A and F of V_2 and V_1 were closed, and ports C and D opened. In this configuration, the sample circulated continuously through the pump, column, and refractometer until the desired degree of separation was achieved. A part of the eluate may be removed at any stage during the recycling procedure by closing ports C and D, and opening ports A and F. One of the two channels of the peristaltic pump serves the recycling circuit and the other channel feeds eluent *via* a 0.22-μm filter to the reference cell of the refractometer (this circuit is not shown in Fig. 1). The type of pump used fulfilled the need of minimum dead volume and yet provided sufficient pressure (~0.1 MPa) to maintain a reasonable flow rate. The dead space at the top of the column was eliminated by the use of adjustable plungers. During long recycling runs some liquid was lost from the circuit and was replaced by air drawn in at leaks on the suction side of the pump. This air eventually passed through the column and into the refractometer causing multiple spikes to appear on the recorder trace. It was found that this loss might be 1–2 mL in the course of a 20-cycle experiment. To counter this, a small reservoir fashioned from a 10-mL syringe and filled with the eluent was fitted *via* a T-junction located between the two valves and made good any losses of liquid from the circuit. To prevent the growth of bacteria in the apparatus, the system was regenerated by elution with 0.5M KOH between runs and all the liquid entering the column did so after passage through a 0.22-μm filter.

One problem frequently encountered during recycling chromatography is that, as the separation develops and the bands separate and broaden, the front of one cycle may catch up with the tail of the previous one. The longer the column is, the greater the number of cycles which could be completed before this occurred. However, back pressure problems were encountered with very long columns, and the 180-cm ones described are a reasonable compromise and allowed the separation of a two-component system in up to 30 or so cycles without the front and tail of the bands intermingling.

Final purification of oligosaccharides. — Traces of a polymeric material were continually released by Zerolit resin, and to remove this and other ionic impurities, solutions of the oligosaccharides were passed through small columns containing 1 mL each of AG 1 (AcO^-) and AG 50W (H^+) ion-exchange resin. The solutions were then freeze-dried.

RESULTS AND DISCUSSION

Preliminary separation of milk oligosaccharides on Zerolit 225 (K^+). — An oligosaccharide fraction of human milk was obtained essentially as described earlier[15]. Milk (1 L), usually from several donors (both secretors and non-secretors),

was dialysed against water (5 × 4-L changes over 7 days) at 4°. The diffusates were passed through a column (30 × 3 cm) of DEAE-cellulose, concentrated to about 75 mL, and kept overnight at 4°. The lactose which crystallised out was filtered off and the volume of the filtrate adjusted to 100 mL. This was then fractionated in two batches of 50 mL on columns (two, each 80 × 5 cm, connected in series) which contained Zerolit 225 X4 (K^+, minus 200 mesh). The columns were eluted with water at 100 mL/h and the eluate was monitored with a differential refractometer. The trace obtained is shown in Fig. 2. Fractions were pooled as indicated by the bars.

Fraction 5. Trisaccharides. — This fraction contained mainly **2** and was further purified as described earlier[15]. In this procedure, the fraction was separated on a column (25 × 2.5 cm) of charcoal which was eluted successively with aliquots (1 L) of water, and then 1, 2.5, 5, and 10% ethanol. Most of the **2** was recovered from (1 L) of water, and then 1, 2.5, 5, and 10% ethanol. Most of **2** was recovered from the 2.5 and 5% ethanol fractions. The 10% ethanol fraction was separated by paper chromatography in solvent *A*. A component having R_{Lac} 0.49 (Solvent *A*) was recovered (yield typically 10 mg/mL of milk), which on chromatography in solvent *B* separated into approximately equal amounts of Trisaccharides I and II (R_{Lac} 0.46 and 0.43, respectively), which were further purified by rechromatography in solvent *B*. Both trisaccharides were shown on analysis to contain two residues of galactose to one of glucose. Methylation analysis of the derived alditols yielded 2,3,4,6-tetra-*O*-methylgalactose and 1,2,3,5,6-penta-*O*-methylglucitol from both compounds. In addition, Trisaccharide I gave 2,4,6-tri-*O*-methylgalactose, and Trisaccharide II 2,3,4-tri-*O*-methylgalactose. The trisaccharides and their alditols were examined by 500-MHz ^{1}H-n.m.r. spectroscopy. The chemical shifts for these compounds are

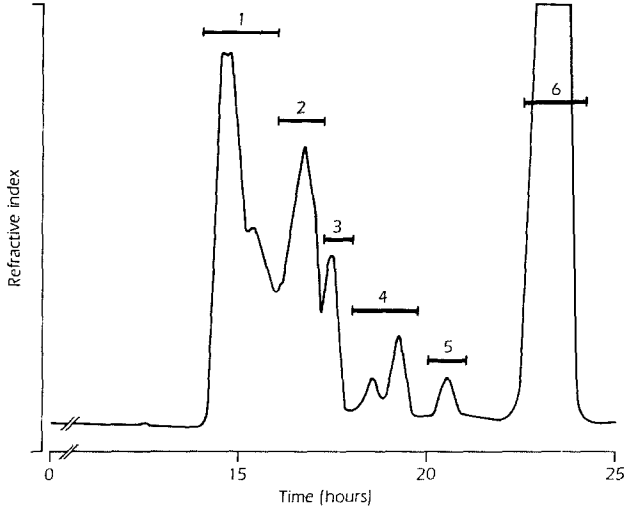

Fig. 2. Preliminary separation of the milk oligosaccharides. The diffusate from 1 L of milk was chromatographed on two columns (80 × 5 cm) of Zerolit 225 (K^+) connected in series and eluted with water at 100 mL/h. The eluate was divided into fractions as indicated by the bars.

TABLE I

^{1}H-CHEMICAL SHIFTS (δ) OF THE STRUCTURAL REPORTER GROUPS OF THE ALDITOLS OF SOME MILK OLIGOSACCHARIDES

Reporter group	*Atom*	*Alditol of*						
		2	**3**	**4**	**5**	**8**	**11**	**14**
α-L-Fuc*p*-(1→2)	H-1	5.320				5.417		
	H-5	4.245				4.212		
	H-6	1.228				1.223		
α-L-Fuc*p*-(1→3)	H-1		5.079			5.069		5.064
to D-Glc	H-5		4.267			4.412		4.276
	H-6		1.214			1.215		1.211
α-L-Fuc*p*-(1→3/4)	H-1						5.131	5.129
to GlcNAc	H-5						4.842	4.832
	H-6						1.174	1.174
β-D-Gal*p*(ext.)	H-1	4.574	4.488	4.616	4.522	4.566	4.463	4.464
	H-2	3.652	3.529	3.206	3.548	3.680	3.497	3.496
	H-3	3.867	3.641	3.667	3.782	3.864		
	H-4	3.906	3.917	3.920	3.921	3.889	3.936	3.913
β-D-Gal*p*(int.)	H-1			4.563	4.448		4.489	4.480
	H-2			3.704	3.532		3.570	3.568
	H-3			3.830	3.659			
	H-4			4.198	3.975		4.151	4.146
D-Glcol	H-2		4.168			4.140		4.147
	H-3		4.066			4.061		4.054
D-GlcNAc	H-1						4.713	4.697
	N*H*Ac						2.023	2.025

recorded in Tables I and II. The shifts for H-1 of the D-galactose residues indicated that all are involved in β-D linkages, these findings being supported by the measured values of the $J_{1,2}$ coupling constants (7.8 Hz). The H-4 shifts of the internal D-galactose residue of Trisaccharide I and its alditol were similar to those observed for other (1→3)-β-D-Gal*p*-(1→4) residues of the milk oligosaccharides series. The ^{1}H-n.m.r. spectrum of the alditol of Trisaccharide I is shown in Fig. 3. On the basis of these findings, Trisaccharide I was determined to be 3′-galactosyllactose (**4**), and Trisaccharide II 6′-galactosyllactose (**5**).

6′-Galactosyllactose (**5**) is a known component of human milk[5], but previously 3′-galactosyllactose (**4**) had only been reported in the milk of marsupials in which it is a major component of the oligosaccharide fraction[18]. Both **4** and **5** have been obtained from culture filtrates of certain fungi[19].

Fraction 4. Tri- and tetra-saccharides. — The major components of Fraction 4 were **3** and **6–8**, and these were separated by recycling chromatography on a column (180 × 2.2 cm) of Zerolit 225 (K^+) using the apparatus shown diagramatically in Fig. 1. The columns were eluted with water at a flow rate of ~40 mL/h. During the first cycle, contaminating pentaoses were removed and some **2** was taken out on the second cycle. The broader appearance of the two major peaks on the first cycle,

TABLE II

^{1}H-CHEMICAL SHIFTS (δ) OF THE STRUCTURAL REPORTER GROUPS OF SOME MILK OLIGOSACCHARIDES

Reporter group	*Atom*	*Oligosaccharide*						
		2	**3**	**4**	**5**	**8**	**11**	**14**
α-L-Fuc*p*-(1→2)	H-1	5.317				5.273		
	H-5	3.938[a]				4.255[a]		
	H-6	1.224[a]				1.262[a]		
α-L-Fuc*p*-(1→3)	H-1(α)		5.389			5.394		5.372
to D-Glc	H-1(β)		5.446			5.451		5.428
	H-5		4.84			4.871[a]		4.82
	H-6(α)		1.193			1.242		1.163
	H-6(β)		1.187			1.240		1.157
α-L-Fuc*p*-(1→3/4)	H-1						5.127	5.135
to D-GlcNAc	H-5						4.830	4.826
	H-6						1.174	1.174
β-D-Gal*p*(ext.)	H-1	4.52	4.437	4.611	4.461	4.492	4.156	4.096
	H-2	3.668	3.50			3.479	3.497	3.496
	H-3							3.653
	H-4		3.898	3.918	3.922			3.782
β-D-Gal*p*(int.)	H-1			4.511	4.489[a]		4.432	4.414
	H-2							3.461
	H-3							
	H-4			4.198	3.968		4.462	4.466
D-Glc	H-1(α)	5.226	5.187	5.224	5.223	5.176	5.219	5.180
	H-1(β)	4.634	4.658	4.667	4.670	4.624	4.662	4.651
	H-2	3.293	[b]	3.286	3.295	[b]	3.277	3.7–3.8
D-GlcNAc	H-1						4.713	4.700[a]
	NHAc						2.022	2.018

[a]Value for the β-anomer of the oligosaccharide is given. [b]Occurs in the skeletal proton region.

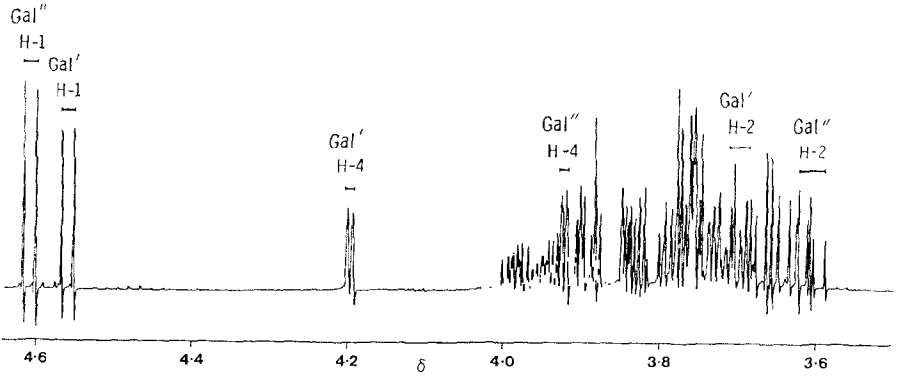

Fig. 3. Resolution-enhanced 500-MHz ^{1}H-n.m.r. spectrum of β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-D-Glcol. Conditions as described in the Experimental section.

compared to subsequent cycles (see Fig. 4A), was due to a perturbance of the flow rate. On the third cycle the two peaks were fully resolved. The faster-moving peak contained **7** and **8**, and the slower-moving **3** and **6**. These mixtures were each satisfactorily resolved into their constituent oligosaccharides by chromatography on a column (180 × 2.2 cm) of Fractogel TSK 40-W(S) as shown in Figs. 4B and 4C.

Fraction 3. Pentaoses. — This was fractionated initially by recycling on a column (80 × 5 cm) of Bio-Gel P-4 (minus 400 mesh). The column was eluted with 1% acetic acid at a flow rate of 40 mL/h, and after seven cycles the pentaoses were separated into two fractions (Fig. 5A), one of which contained practically pure **9** and the other a mixture of **10** and **11**. Traces of other unidentified sugars having the chromatographic mobility of pentaoses were removed from the fraction containing **9** by recycling on the 180 × 2.2 cm Zerolit column (separation not shown). The

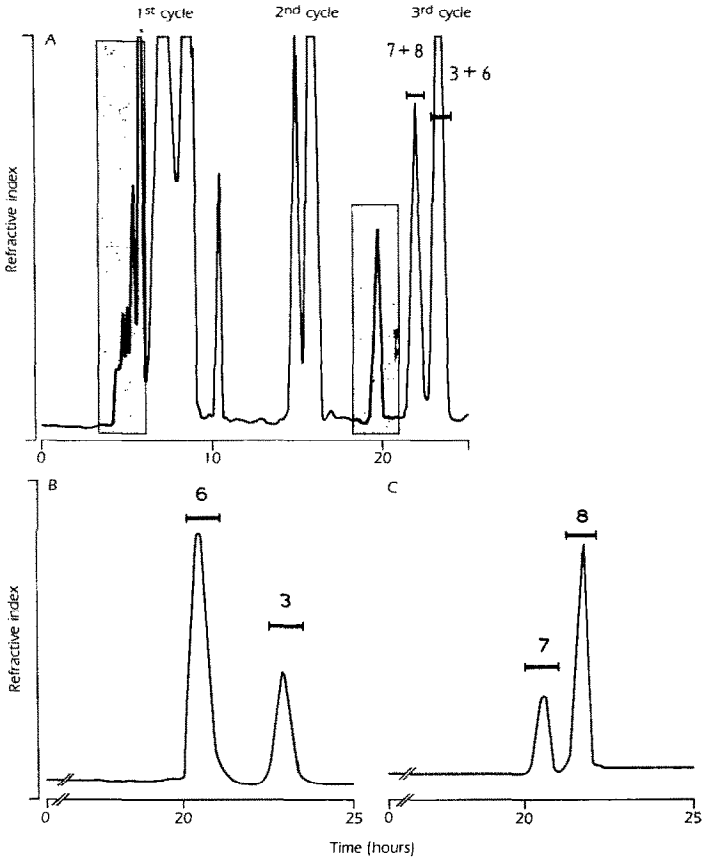

Fig. 4. Resolution of the tetraose fraction of human milk. (A) Fraction 4 from Fig. 2 was subjected to recycling chromatography on a column (180 × 2.2 cm) of Zerolit 225 (K^+), eluted with water at 40 mL/h. Eluate corresponding to the hatched regions was removed during the first and second cycles. (B) Separation of **3** and **6** on a column (180 × 2.2 cm) of Fractogel TSK 40W(S) eluted with water at 15 mL/h. (C) Resolution of **7** and **8**; conditions as described for B.

mixture of **10** and **11** was then resolved by recycling on the Zerolit column (Fig. 5B). The separation ws usually completed within 15–25 cycles. Typically 0.5 g of a mixture may be separated in ~20 cycles (Fig. 5B). Although resolution of the alditols of **10** and **11** by t.l.c. after acetylation has been reported[20], no method for the separation of mixtures of **10** and **11** has previously been described. Compound **11** is usually obtained from the milk of very rare Le^{a-b-} non-secretor individuals who fail to synthesise **10**, which itself may be obtained by fractional precipitation of a mixture of **10** and **11** with acetone[5]. The technique of separation described here means that pooled milk, or milk from the more commonly encountered Le^{a+b-} and Le^{a-b+} donors becomes a source of reference oligosaccharides containing the Le^a determinant (**10**) and the β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-β-D-Glc*p*NAc terminal group of **11** that is sometimes called the Le^x, LeX, or SSEA-1 determinant.

Fraction 2. Hexaoses. — The hexaose fraction could also be resolved by recycling on the Zerolit column (180 × 2.2 cm), as shown in Fig. 6 for the hexaose fraction of pooled milk from non-secretors. Traces of pentaoses and sugars having a higher mol. wt. than hexasaccharides were removed from the mixture on the 2nd and 5th cycles. As recycling progressed, a minor peak was collected during the 12th

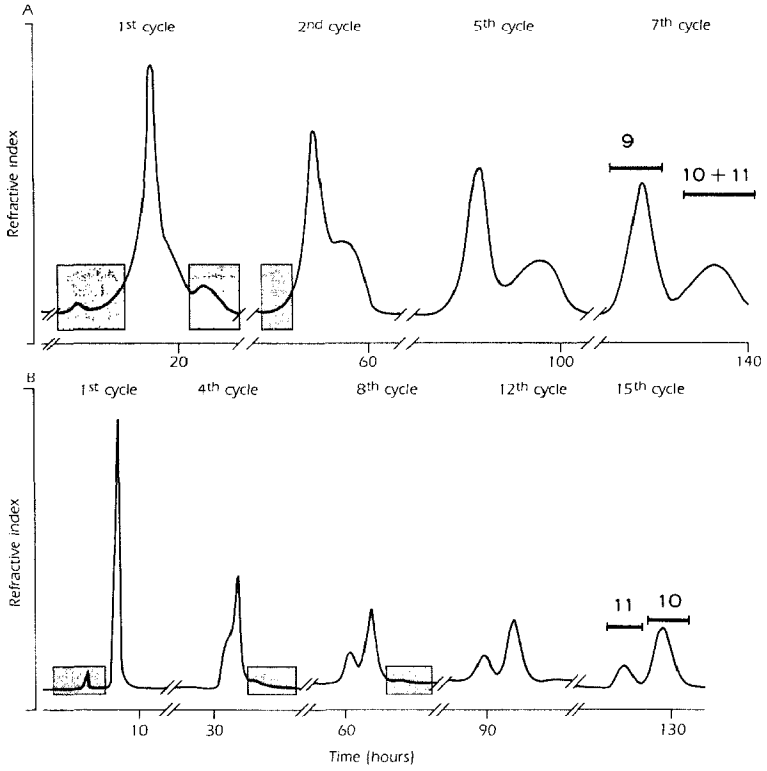

Fig. 5. Separation of the pentaose fraction: (A) Recycling chromatography of Fraction 3 from Fig. 2 on a column (80 × 4.4 cm) of Bio-Gel P-4, eluted with 1% acetic acid at 40 mL/h. (B) Resolution of **10** and **11** recycling chromatography on a column of Zerolit 225; conditions as described for Fig. 4A.

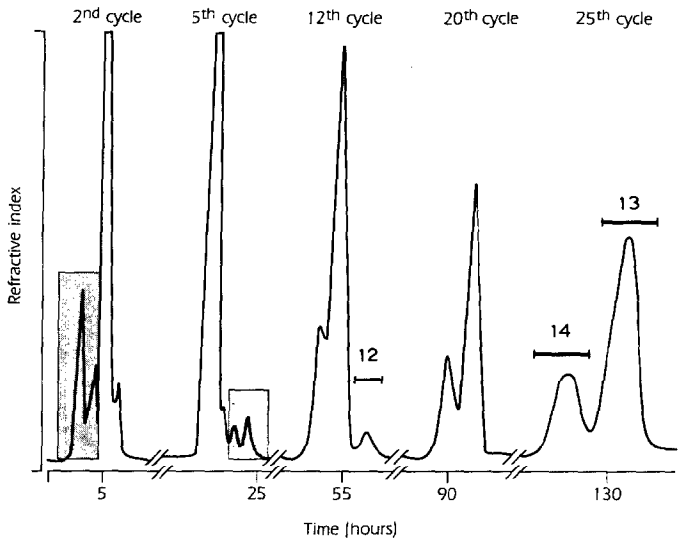

Fig. 6. Separation of the hexaoses. The hexaose fraction from non-secretor milk was resolved by recycling on a column of Zerolit 225 with conditions as described for Fig. 4A. Eluate from the hatched regions of the chromatogram was removed during the 2nd and 5th cycles. A small amount of **12** was recovered in the 12th cycle, and resolution of **13** and **14** was complete after 25 cycles.

cycle and subsequently identified as **12**. The reason for the isolation of this hexaose from non-secretor milk is unclear. After removal of **12**, the rest of the mixture was left to complete 25 cycles when it separated into two components subsequently identified as **13** and **14**. The latter compound, obtained in a yield of 10–20 mg/L of milk, has not previously been reported as a constituent of human milk, although an unresolved mixture of this compound with **13** has been isolated from human urine obtained during pregnancy and lactation[21], and more recently it has been found in platypus milk[22].

Identification of compound **14**. — Analysis showed that this compound contains fucose, galactose, 2-acetamido-2-deoxy glucose, and glucose in the ratio 193:209:97:100. After methylation and hydrolysis of the alditol, 2,3,4-tri-*O*-methylfucose, 2,3,4,6-tetra-*O*-methylgalactose, 2,4,6-tri-*O*-methylgalactose, 2-amino-2-deoxy-6-*O*-methylglucose, and 1,2,5,6-tetra-*O*-methylglucitol were identified. The tetrasaccharide backbone of the oligosaccharide was shown to be **7** rather than **6** after removal of the fucosyl groups from the alditol by hydrolysis with 10% acetic acid for 1 h at 100°, followed by h.p.t.l.c. of the product. The alditols of **6** and **7** were readily separated by this procedure.

The 500-MHz ^{1}H-n.m.r. spectra were recorded for both **14** and its alditol, and these showed that both samples contain no more than minor amounts of contaminants; the spectrum of the alditol is shown in Fig. 7. The chemical shifts of the L-fucosyl group and D-glucitol residue from the β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-D-Glcol region of the alditol show homologies with the model compound, alditol of

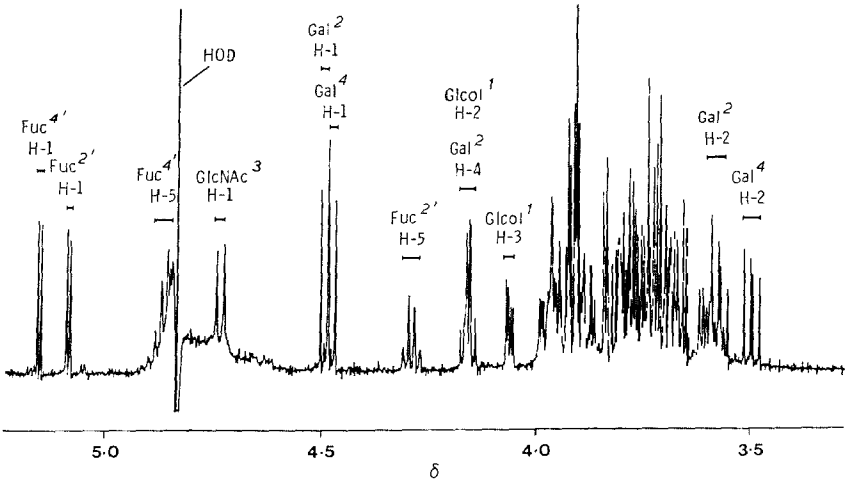

Fig. 7. Resolution-enhanced 500-MHz ^{1}H-n.m.r. spectrum of the alditol of **14**.

3, in particular the signals for H-2 and H-3 of the D-glucitol residue occurred outside the bulk of the skeletal proton signals at δ 4.147 and 4.054 (*cf.*, δ 4.168 and 4.066 for the alditol of **3**), and the H-1, H-5, and methyl groups chemical shifts of the L-fucosyl groups at δ 5.064, 4.276, and 1.1211 were close to those recorded for the alditol of **3** (δ 5.079, 4.267, and 1.215). The shifts for the β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→3)]-D-GlcNAc nonreducing end group (H-1 of Fuc δ 5.129, H-5 of Fuc 4.832, H-1 of GlcNAc 4.697, H-1 of Gal 4.480, and N*H*Ac 2.025) are almost the same as those observed for the same protons in the alditol of **11** (δ 5.131, 4.842, 4.713, 4.489, and 2.023, respectively) and clearly different from those in the alditol of **10** (δ 5.029, 4.870, 4.697, 4.508, and 2.032, respectively)[23]. The chemical shifts and the coupling constants for the fucopyranosyl groups showed these to be involved in α linkages, and corresponding values for the 2-acetamido-2-deoxy-D-glucopyranosyl and D-galactopyranosyl residues indicated a β configuration.

^{1}H-N.m.r. spectra of other oligosaccharides. — The chemical shifts of **2, 3, 8,** and **11** are included in Tables I and II for comparison purposes. The ^{1}H-n.m.r. data for many of the other milk oligosaccharides have been given elsewhere[23-25]. Those prepared as described above were routinely examined by 500-MHz ^{1}H-n.m.r. spectroscopy both to check their identity and to assess their purity, a matter of some importance for their use as acceptor substrates in glycosyltransferase studies. In these, the oligosaccharide is used in a large (generally >100-fold) molar excess over a [^{14}C]nucleotide sugar donor, so that small amounts of impurities which are good acceptors can interfere significantly in transferase experiments.

Basis of the separation achieved on cation-exchange resins (K^+). — The ability of recycling chromatography on cation-exchange resins (K^+) to separate isomeric mixtures of oligosaccharides is amply demonstrated in this paper. The technique allowed the separation of about 1 g of such mixtures on relatively modest-sized columns for only a small expenditure of operator time. Furthermore, the method

was capable of performing separations of mixtures of isomers that have proved difficult or impossible to carry out by established procedures. Of the pairs of isomers separated, *i.e.*, **6** and **7**, **9** and **10**, and **13** and **14**, the one containing the Type 1 backbone, β-D-Gal*p*-(1→3)-D-Glc*p*NAc, emerged first from the column. The mechanism of the separation achieved on the K^+ resins is not apparent. Ligand exchange, as proposed by Goulding[26], may be one of the factors operating, but as there is no difference in the number of favourable axial–equatorial or axial–equatorial–axial combinations of hydroxyl groupings between **6** and **7**, this effect is likely to be of minor importance. It seems more probable that the different mobilities of the isomers results from a combination of subtle differences in the shape of the molecules and relative arrangements of their hydrophobic and hydrophilic sites, and the interaction of these with the resin matrix.

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