THE BIOSYNTHESIS OF A BLOOD GROUP B ACTIVE TETRASACCHARIDE

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1. Introduction

The hypothesis [1, 2] that the genes responsible for the blood group characters associated with the ABO and Lewis systems specify the formation, or control the activity, of glycosyltranferases is supported by experiments with particle-bound enzyme preparations from human tissues synthesising blood group active glycoproteins [3–8] and soluble enzymes occurring in human milk [9–13]. Gastric mucosal linings and submaxillary glands from group B and AB subjects contain a particle-bound α-D-galactosyltransferase that is absent from tissues of group A or O subjects [3–5]. This enzyme transfers D-galactose from uridine diphosphate (UDP) D-galactose to oligosaccharides containing at the terminal non-reducing end the H-active structure, α-L-fucosyl-(1→2)-D-galactose. The anomeric linkage of the transferred galactose was established as α by enzymic methods [3, 4, 9] and the positional linkage was inferred to be (1→3) [4] because the compound formed by transfer of D-^{14}C-galactose to the disaccharide *O*-α-L-fucosyl-(1→2)-D-galactose gave, on mild acid hydrolysis, a radioactive compound that co-chromatographed with the B-active disaccharide [14] *O*-α-D-galactosyl-(1→3)-D-galactose. Unequivocal proof of the positional linkage of the added galactose, and evidence that the product was serologically active, were, however, lacking. This paper describes the chemical characterisation and serological properties of the tetrasaccharide synthesised from UDP-D-galactose and 2′-fucosyl-lactose (*O*-α-L-fucosyl-(1→2)-β-D-galactosyl-(1→4)-D-glucose) [15] by a particle-bound enzyme from human group B stomach linings. This tetrasaccharide has the structure *O*-α-D-galactosyl-(1→3)-[*O*-α-L-fucosyl-(1→2)]-β-D-galactosyl-(1→4)-D-glucose.

2. Materials and methods

Unlabelled UDP-D-galactose was purchased from Sigma, London and UDP-D-^{14}C-galactose (240 mCi/mmole) from the Radiochemical Centre, Amersham. 2′-Fucosyl-lactose was a gift from Dr.A.Gauhe. Methyl-3, 4, 6-tri-*O*-methyl-D-galactoside and methyl-2, 4-di-*O*-methyl-D-galactoside were supplied by the late Professor R.Kuhn, methyl-4, 6-di-*O*-methyl-D-galactoside and methyl-2, 3, 6-tri-*O*-methyl-D-galactoside by Dr.G.O.Aspinall and methyl-2, 3-di-*O*-methyl-D-galactoside by Professor T.Reichstein. Methyl-2, 3, 4, 6-tetra-*O*-methyl-D-galactoside and methyl-2, 3, 6-tri-*O*-methyl-D-glucoside were prepared by methylation of lactose, methyl-2, 3, 4-tri-*O*-methyl-L-fucoside and methyl-3, 4, 6-tri-*O*-methyl-D-galactoside by methylation of 2′-fucosyl-lactose, methyl-2, 3, 4-tri-*O*-methyl-D-galactoside by methylation of *O*-β-(*N*-acetyl)-D-glucosaminyl-(1→6)-D-galactose [16], and methyl-2, 4, 6-tri-*O*-methyl-D-galactoside by methylation of the trisaccharide 3-*O*-α-D-galactotriose, supplied by Dr.J.Koscielak.

Paper chromatography was carried out in ethyl acetate-pyridine-water (solvent *a*, 10:4:3, by vol.; solvent *b*, 2:1:2 by vol. upper phase) on Whatman No. 40 paper. Sugars were visualised with alkaline silver nitrate [17] and the radioactive peaks were detected with a 7201 Packard Radiochromatogram Scanner.

Gas-liquid chromatography was carried out in a Pye "series 104" chromatograph with a dual flame ionisation detector. For estimation of the component sugars the tetrasaccharide was hydrolysed with 0.5 N HCl at 100° for 4 hr and the alditol acetates, prepared by the method of Lehnhardt and Winzler [18],

were separated on a coiled glass column (5 ft × 4 mm) packed with 3% ECNSS-M on 80–100 mesh Gas-Chrom Q (Applied Science Laboratories Inc.) with a temperature programme of 2°/min from 155° to 195° [19]. Standard sugars were subjected to the same hydrolysis conditions as the tetrasaccharide, and response factors for each sugar were calculated relative to xylitol, which was added before hydrolysis to both the tetrasaccharide and the solution containing the standard mixture of sugars.

Oligosaccharides were methylated by a micro-adaptation [20] of the methods of Hakomori [21] and Choi and Carubelli [22]. The methyl glycosides were separated by gas-liquid chromatography on coiled glass columns (5 ft × 4 mm) packed with (a) 3% EGA on Gas-Chrom Q, (b) 3% EGSS-X on Gas-Chrom Q and (c) 10% Carbowax 20M on Gas-Chrom CLH. All the column fillings were from Applied Science laboratories Inc., and the Gas-Chrom was 80–100 mesh. The methylated sugars were identified by comparison of their retention times with those of authentic samples chromatographed under the same conditions.

The enzyme source for the synthesis of the tetrasaccharide was a particulate preparation isolated from human group B stomach mucosal lining [3]. The particles from 10 g wet tissue were suspended in 1.0 ml of 0.05 M tris-HCl pH 7.2 (containing 0.05 M mercaptoethanol). The suspension was added to 10 ml of acetone at −15°, the mixture was stirred vigorously and left to stand at 0° for 15 min. The insoluble material was spun down, resuspended in 3 ml of acetone at −15°, respun and washed once more with 3 ml of cold acetone and dried. The acetone powder (50 mg) was incubated for 16 hr at 37° with: 10 μmoles ATP, 10 μmoles $MnCl_2$, 5 μmoles UDP-^{14}C-galactose (1,500,000 cpm), 5 μmoles 2′-fucosyl-lactose, and 750 μl 0.05 M tris-HCl pH 7.2 containing 0.05 M mercaptoethanol (total volume 1.4 ml). The neutral sugars in the incubation mixture, separated from the charged components by electrophoresis as described previously [3], were chromatographed in solvent *a* and the radioactive component with an R_f value corresponding to a tetrasaccharide was purified by repeated chromatography in solvent *b*. Yield of tetrasaccharide 1.5 mg (705,000 cpm).

An incubation mixture containing all the components except 2′-fucosyl-lactose and UDP-^{14}C-galactose was prepared to detect any sugars that might have arisen from the enzyme preparation, and which migrated on chromatography in the tetrasaccharide area. It was incubated and worked up in the same way as the mixture used to synthesise the tetraccharide and the corresponding area of the chromatogram was eluted, the eluate concentrated and included in the methylation and serological experiments.

The serological activity of the synthesised tetrasaccharide was measured with human anti-A and anti-B reagents and with anti-H serum prepared in the rabbit [23].

3. Results

3.1. *Identification and estimation of the sugar components of the tetrasaccharide*

The tetrasaccharide (45 μg; 21,000 cpm) was hydrolysed for 4 hr with 0.5 N HCl and the products, after removal of the acid, were examined by paper chromatography in solvent *a*. Development with alkaline silver nitrate gave only three spots corresponding in R_f to galactose, glucose and fucose. Only one radioactive area, corresponding to the galactose spot, was detected in the Radiochromatogram Scanner.

Xylitol (25 μg) was mixed in 100 μl of water with 150 μg of the tetrasaccharide and the mixture was heated for 4 hr at 100° with an equal volume of 1.0 N HCl. Alditol acetates were prepared from the hydrolysis products and separated by gas-liquid chromatography. Three peaks were detected, in addition to the xylitol acetate peak, with retention times corresponding to the alditol acetates of fucose, galactose and glucose. Integration of the peaks, and calculation of the response factor of each sugar relative to xylitol, indicated that the tetrasaccharide had a fucose-galactose-glucose ratio of 1.0:2.0:0.9.

3.2. *Methylation analysis*

The tetrasaccharide (500 μg) was methylated [20–22] and the product was repeatedly dissolved in chloroform, extracted with water and dried *in vacuo*. The resulting residue gave a single spot when examined by thin layer chromatography in chloroform-methanol-water (90:10:1), indicating complete methylation. The methylated tetrasaccharide was hydrolysed with 0.5 N methanolic HCl for 16 hr at 75–80°. The products, examined by gas liquid chromatography, gave six

Table 1
Relative retention times of peaks given on gas chromatography of various methylated sugars and the methylated products from the synthesised tetrasaccharide.

Sample	Relative retention times of peaks *		
	EGA column at 160°	EGSS-X column at 160°	Carbowax 20M column at 170°
Methyl-2, 3, 4-tri-*O*-methylfucoside	0.40	0.20	1.00
Methyl-2, 3, 4, 6-tetra-*O*-methylgalactoside	1.00	1.00	
Methyl-2, 3, 6-tri-*O*-methylglucoside	1.96 2.76	1.92 2.75	2.08 2.71
Methyl-3, 4, 6-tri-*O*-methylgalactoside	2.48 4.00	2.75 4.33	
Methyl-2, 3-di-*O*-methylgalactoside		6.33 8.50 11.1	
Methyl-2, 4-di-*O*-methylgalactoside	10.0 11.7	11.1 13.0	
Methyl-4, 6-di-*O*-methylgalactoside	5.35 9.45	5.40 10.4	5.20 9.50
Methylated products from tetrasacchairde	0.40	0.20	
	1.00	1.00	1.00
	1.96 2.76	1.92 2.83	2.05 2.71
	5.35 9.40	5.40 10.4	5.20 9.50

* Relative to methyl-2, 3, 4, 6-tetra-*O*-methylgalactoside.

distinct peaks (table 1) with retention times corresponding to those given by the α- and β-anomers of methyl-2, 3, 4-tri-*O*-methylfucoside (peak 1), methyl-2, 3, 4, 6-tetra-*O*-methylgalactoside (peak 2), methyl-2, 3, 6-tri-*O*-methylglucoside (peaks 3 and 4) and methyl-4, 6-di-*O*-methylgalactoside(peaks 5 and 6).

The area corresponding to the tetrasaccharide was eluted from the chromatogram of a control incubation mixture from which UDP-^{14}C-galactose and 2'-fucosyllactose were omitted and the dried eluate subjected to methylation and methanolysis under the same conditions as the radioactive tetrasaccharide. No methylated sugars were detected on gas chromatography of the control preparation, indicating that the methylated derivatives did not arise from oligosaccharides released from the particulate enzyme preparation.

The three authentic methyl-di-*O*-methylgalactosides were clearly separated (table 1) and the identity of the retention times of the two peaks from the tetrasaccharide with those given by the 4, 6 derivative is consistent with the interpretation that the subterminal galactose residue in the tetrasaccharide is substituted at the 2-position with fucose and at the 3-position with galactose. However, as authentic specimens of methyl 3, 6- and 3, 4-di-*O*-methylgalactosides were not available for comparison, the tetrasaccharide was hydrolysed under conditions that preferentially released fucose in order that the methylation products of the resultant trisaccharide could be compared with authentic methyl tri-*O*-methylgalactosides. Hydrolysis of the tetrasaccharide in 0.5 N acetic acid at 100° for 16 hr removed about 40% of the fucose. The newly formed trisaccharide was separated from the released fucose and unchanged tetrasaccharide by paper chromatography in solvent *a* and purified by paper chromatography in solvent *b*. Hydrolysis of the trisaccharide with 0.5 N HCl for 4 hr yielded only two products with R_f values on paper chromatography corresponding to galactose and glucose. The trisaccharide was methylated, methanolysed and the products examined by gas chromatography (table 2). The relative rentention times of the products corresponded to the peaks given by authentic samples of the α- and β-anomers of (1) methyl-2, 3, 4, 6-tetra-*O*-methylgalactoside (2) methyl-2, 3, 6-tri-*O*-methylglucoside and (3) methyl-2, 4, 6-*O*-tri-*O*-methylgalactoside. The identification of the methyl tri-*O*-methylgalactoside as the 2, 4, 6-derivative thus supported the inference from the methylation analysis of the tetrasaccharide that the terminal galactose is linked to 3-position of the subterminal galactose residue.

3.3. *Serological examination of the tetrasaccharide.*

The tetrasaccharide was tested for haemagglutina-

Table 2
Relative retention times of peaks given on gas chromatography of the methylated products from the trisaccharide

Sample	Relative rentention times of peaks *				
	EGA column at 140°		Carbowax 20M column at 170°		
Methyl-2, 3, 4, 6-tetra-*O*-methylgalactoside	1.00	1.10		1.00	
Methyl-2, 3, 6-tri-*O*-methylglucoside	2.18	3.22	2.08		2.71
Methyl-3, 4, 6-tri-*O*-methylgalactoside	2.82	4.80	2.32		3.92
Methyl-2, 3, 6-tri-*O*-methylgalactoside	1.97	3.18	1.83		2.48
Methyl-2, 4, 6-tri-*O*-methylgalactoside	2.53	3.13	2.29		2.50
Methyl-2, 3, 4-tri-*O*-methylgalactoside	2.87	4.10	2.62	3.34	4.22
Methylated products from trisaccharide	1.00	1.10		1.00	
	2.16	3.16	2.08		2.71
	2.55	3.16	2.29		2.52

* Relative to 1st peak of methyl-2, 3, 4, 6-tetra-*O*-methylgalactoside.

Table 3
Haemagglutination inhibition tests with the synthesised tetrasaccharide.

	Minimum concentration of substance giving complete inhibition (μg/100 μl)		
	Anti-B serum	Anti-A serum	Anti-H serum
Synthesised tetrasaccharide			
α-Gal-(1→3)-[α-Fuc-(1→2)]-β-Gal-(1→4)-Glc	62	> 2000	> 2000
Tetrasaccharide isolated from B substance			
α-Gal-(1→3)-[α-Fuc-(1→2)]-β-Gal-(1→4)-GNAc	62	> 2000	> 2000
2′-Fucosyllactose			
α-Fuc-(1→2)-β-Gal-(1→4)-Glc	> 2000	> 2000	62

Abbreviations: Gal = D-galactosyl; Fuc = L-fucosyl
Glc = D-glucose; GNAc = *N*-acetyl-D-glucosamine

tion with human anti-B, human anti-A and rabbit anti-H serum (table 3). No inhibition was observed in the anti-A and anti-H systems but the synthesised tetrasaccharide inhibited anti-B serum to the same extent as the B-active tetrasaccharide isolated from human B specific glycoprotein [24]. The two tetrasaccharides differ in that the reducing sugar in the synthesised compound is glucose, whereas in the B-active tetrasaccharide it is *N*-acetylglucosamine. The acceptor trisaccharide, 2′-fucosyl-lactose, inhibited the anti-H serum but this inhibition was completely masked by the addition of the non-reducing galactosyl residue. The eluate from the tetrasaccharide area of the chromatogram of the control incubation mixture did not give inhibition in any of the haemagglutination systems.

4. Discussion

The identification of fucose, galactose and glucose in the ratio of 1:2:1 in the radioactive product synthesised from UDP-^{14}C-galactose and 2′-fucosyl-lactose by a particulate enzyme preparation from a blood group B subject confirms that a single galactose residue is

tranferred to the trisaccharide acceptor to form a tetrasaccharide. The presence of methyl-4, 6-di-*O*-methylgalactoside in the methylated products of the tetrasaccharide, and of methyl-2, 4, 6-tri-*O*-methylgalactoside in the methylated products of the trisaccharide formed by the release of fucose, proves that the β-galactosyl residue in 2-fucosyl-lactose is substituted at the 3-position by the transferred galactose unit. The serological activity of the tetrasaccharide in B-anti-B inhibition tests, and the previous confirmation of the anomeric linkage of the transferred galactose as α [4], is in agreement with the assignment of the following structure to the synthesised tetrasaccharide:

α-D-Galactosyl-(1→3)-β-D-Galactosyl-(1→4)-D-Glucose

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α-L-Fucosyl

The characterisation of this tetrasaccharide confirms that the enzyme associated with the blood group B character is a uridine disphosphate D-galactose: [α-1, 2-L-fucosyl]-β-D-galactosylsaccharide α-3-galactosyltransferase.

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