A Serologically Active Tetrasaccharide Obtained from a Human Blood-Group B-Active Glycoprotein by Partial Acid Hydrolysis

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A B-active disaccharide (Painter, Watkins & Morgan, 1962) and two active trisaccharides (Painter, Watkins & Morgan, 1963) have been recovered from the partial acid hydrolysis products of a purified group B substance obtained from an ovarian cyst fluid of human origin. The disaccharide and two trisaccharides were identified as 3-*O*- α -D-galactosyl-D-galactose, *O*- α -D-galactosyl-(1 $\rightarrow$ 3)-*O*- β -D-galactosyl-(1 $\rightarrow$ 3)-*N*-acetyl-D-glucosamine and *O*- α -D-galactosyl-(1 $\rightarrow$ 3)-*O*- β -D-galactosyl-(1 $\rightarrow$ 4)-*N*-acetyl-D-glucosamine respectively. From the products of the partial acid hydrolysis of B substance a serologically active tetrasaccharide has now been obtained, and its properties and structure are here recorded.

Blood-group B substance [no. 531, 15 g.; analytical values (for methods see Pusztai & Morgan, 1964): fucose, 22%; reducing sugars, after acid hydrolysis, 51%; amino sugars, 27%; amino acids, 19%; galactosamine/glucosamine molar ratio, 1:2.8] was dissolved in 900 ml. of 0.04*N* water-soluble polystyrenesulphonic acid resin (Painter & Morgan, 1961) and continuously hydrolysed and dialysed, as described by Marr, Donald, Watkins & Morgan (1967). The diffusible products were recovered by evaporation of the diffusate to a small volume at a temperature not above 30°, and drying from the frozen state. The material (11.1 g.) was dissolved in water (50 ml.) and passed through a column (82 cm. $\times$ 4.5 cm.) of Sephadex G-15, eluted with water and separated into fractions. Every third fraction was examined by thin-layer chromatography (with propan-1-ol-ethyl acetate-water, 6:1:3, by vol.) to determine the general pattern of carbohydrate elution. The fractions were examined by paper chromatography in solvent mixture *a* (ethyl acetate-pyridine-water, 10:4:3, by vol.) and pooled according to their approximate size as estimated by their R_{lactose} values. Material having R_{lactose} 0.1-1.2 was again passed through a column of Sephadex G-15, and fractions having R_{lactose} values from 0.1 to 1.0 were pooled and recovered. The oligosaccharide mixture (3.4 g.) was further fractionated on a charcoal-Celite column (44 cm. $\times$ 3 cm.) by using a stepwise development with aqueous ethanol, adjusted to pH 3 with formic acid (Taylor & Whelan, 1962), within the range 0-30% (v/v) ethanol. The fractions collected with 10% (v/v) ethanol were neutralized with Permutit

De-Acidite FF.1P (CO_3^{2-} form), concentrated under reduced pressure and dried from the frozen state. The material (160 mg.) was resolved into several components by paper chromatography on Whatman no. 40 paper with solvent *b* (ethyl acetate-pyridine-water, 2:1:2, by vol.). One component (23 mg.) appeared to be homogeneous, but on paper electrophoresis in borate buffer at pH 10 (Foster, 1957) was resolved into three components. Further chromatography of this substance on a column (158 cm. $\times$ 1 cm.) of Bio-Gel P-2 eluted with water gave several fractions, which yielded 7.7 mg. of an electrophoretically homogeneous substance with the following properties: $[\alpha]_{\text{D}} + 81^\circ$ (*c* 1.0 in water), M_{G} 0.57 in borate buffer at pH 10, R_{lactose} 0.26 in solvent *a* and R_{lactose} 0.34 in solvent *b*. The substance gave a strong indirect Ehrlich reaction (Aminoff, Morgan & Watkins, 1952), and on complete hydrolysis with 0.5*N*-HCl at 100° for 17 hr. yielded only galactose (65%) and glucosamine (19.6%). These values indicated that the two sugars were present in the molar ratio 3.00:0.92. Reduction of the substance with NaBH_4 followed by acid hydrolysis changed the galactose/glucosamine molar ratio to 2.00:0.86. The analytical values and chromatographic behaviour indicated that the substance was a tetrasaccharide.

The tetrasaccharide on heating in aq. 50% (v/v) methanol containing triethylamine (5%, v/v) at 75° for 1 hr. gave products, two of which on examination by paper chromatography with solvent *a* were considered to be galactose and the chromogen (Kuhn & Krüger, 1956, 1957) derived from lacto-*N*-biose I [*O*- β -D-galactopyranosyl-(1 $\rightarrow$ 3)-*N*-acetyl-D-glucosamine]. Other unidentified substances were also present.

Partial hydrolysis of the tetrasaccharide by 2*N*-acetic acid at 100° for 17 hr. gave substances that were indistinguishable on paper chromatography (solvents *a* and *b*) from (a) unchanged tetrasaccharide, (b) *O*- α -D-galactopyranosyl-(1 $\rightarrow$ 3)-D-galactose, (c) lacto-*N*-biose I, (d) lacto-*N*-biose II [3-*O*-(*N*-acetyl- β -D-glucosaminopyranosyl)-D-galactose], (e) galactose and (f) *N*-acetylglucosamine. Unidentified substances with R_{lactose} values (solvent *a*) 0.40 and 0.64 were also present. Substances (a), (c), (d) and (f) and the unidentified substances gave a positive indirect Ehrlich reaction.

The tetrasaccharide was incubated (37° for 18 hr.)

Table 1. *Oxidation of the B-active tetrasaccharide with 25 mM-sodium periodate at pH 5 and 18–20°*

Oxidation time	Mol.prop. relative to tetrasaccharide			Theory
	Experimental			
	1 hr.	3 hr.	20 hr.	
Periodate reduced	2.4	3.2	3.1	3.0
Formic acid formed	0.85	1.1	1.3	1.0
Formaldehyde liberated	—	0.2	—	0.0
Formaldehyde liberated from reduced tetrasaccharide	—	2.1	—	2.0

with an α -galactosidase preparation obtained from *Trichomonas foetus* (Watkins, 1959). Galactose was split off from the tetrasaccharide and from 3-*O*- α -galactosyl-D-galactose and melibiose [6-*O*-(α -D-galactopyranosyl)-D-glucose]. A preparation of β -galactosidase from the same organism had no action on the tetrasaccharide or on melibiose, but rapidly hydrolysed lactose [4-*O*-(β -D-galactopyranosyl)-D-glucose].

The tetrasaccharide (0.4 mg.) was methylated with methyl iodide in dimethylformamide and BaO-Ba(OH)₂·8H₂O (Kuhn, Baer & Seeliger, 1958; Kuhn & Trischmann, 1961), and the methylated substance was hydrolysed with 0.5N-H₂SO₄ for 18 hr. at 100°. The acid was removed with BaCO₃ followed by centrifugation. The supernatant was passed through a column of Zeo-Karb 225 (H⁺ form) to adsorb the amino sugar methyl ethers; the neutral methyl ethers, recovered from the eluate, were converted into the methyl glycosides with 0.5N-HCl in methanol at 70–80°. Methyl 2,3,4,6-tetra-*O*-methylgalactoside(s) and methyl 2,4,6-tri-*O*-methylgalactoside(s) were identified on gas-liquid chromatography by comparison with authentic specimens.

The amino sugar methyl ethers were recovered from the Zeo-Karb resin by elution with 2N-HCl. 4,6-Di-*O*-methylglucosamine was identified when the washings were concentrated and examined on an amino acid analyser (Heyns & Kiessling, 1967). The methylation results indicate that in the tetrasaccharide the sugar units are glycosidically linked to position 3 of the next residue.

Oxidation of the tetrasaccharide with NaIO₄ (Belcher, Dryhurst & MacDonald, 1965*a,b*) gave values (Table 1) for the consumption of periodate and for the production of formic acid and formaldehyde close to the values expected for a (1→3)-linked tetrasaccharide. After the tetrasaccharide was reduced with NaBH₄ the subsequent oxidation of the glycitol produced 2.1 mol.prop. of formaldehyde.

On the evidence given it is concluded that the tetrasaccharide is *O*- α -D-galactopyranosyl-(1→3)-*O*- β -D-galactopyranosyl-(1→3)-*O*-(*N*-acetyl- β -D-glucosaminopyranosyl)-(1→3)-D-galactose.

On a molar basis the tetrasaccharide inhibits B-specific haemagglutination to the same extent as the disaccharide *O*- α -D-galactopyranosyl-(1→3)-D-galactose (Painter *et al.* 1962).

The isolation of the tetrasaccharide from the acid hydrolysis products of a purified group-specific B substance extends the structural conclusions reached earlier, and indicates that this serologically active tetrasaccharide is a non-reducing end fragment having only (1→3)-glycosidic linkages and almost certainly comprises part only of a longer carbohydrate chain. The three sugars at the reducing end of the tetrasaccharide are in the same sequence and similarly joined by (1→3)-glycosidic bonds as are the sugars in the main chain of the Leb^b-active fucose-containing pentasaccharide obtained from H substance (Marr *et al.* 1967).

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