Short Communication

A Trisaccharide, *O*- β -D-Galactopyranosyl-(1 $\rightarrow$ 3)-*O*-(*N*-acetyl- β -D-glucosaminopyranosyl)-(1 $\rightarrow$ 4)-D-galactose, Obtained from Human Blood-Group H Substance

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Since the first isolation and identification of serologically active and inactive oligosaccharides from human blood-group A substance (Côté & Morgan, 1956; Cheese & Morgan, 1961) many similar fragments have been recovered from each of the specific substances associated with the *ABO*, *H* and *Le* genetic systems (cf. Morgan, 1964; Watkins, 1966). The carbohydrate fragments identified contain some or all of the four sugars L-fucose, D-galactose, *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine. The trisaccharide sequence D-galactose-*N*-acetyl-D-glucosamine-D-galactose is known to occur with (1 $\rightarrow$ 3)-(1 $\rightarrow$ 3)-, (1 $\rightarrow$ 4)-(1 $\rightarrow$ 3) and (1 $\rightarrow$ 4)-(1 $\rightarrow$ 6)-glycosidic linkages (cf. Watkins, 1966; Lloyd & Kabat, 1967; Aston, Donald & Morgan, 1968). The isolation from a preparation of H substance of a trisaccharide composed of the same three sugars joined in the same order by (1 $\rightarrow$ 3)- and (1 $\rightarrow$ 4)-linkages reveals the presence of yet a different pair of bond sequences and forms the subject of this paper.

Blood-group specific H substance from ovarian cyst no. 511 (15g.) was dissolved in about 900 ml. of a water-soluble non-diffusible polystyrenesulphonic acid (0.04N, pH 2.1) as described by Painter & Morgan (1961) and continuously hydrolysed and dialysed until the release of diffusible fragments had almost ceased. The total diffusible material (10.4g.) was recovered from the concentrated diffusate by drying from the frozen state and chromatographed in portions on a column (82 cm. $\times$ 3.5 cm.) of Sephadex G-10 eluted with water. The fractions obtained were examined for carbohydrate fragments by chromatography on Whatman no. 1 paper developed with the upper phase of ethyl acetate-pyridine-water (2:1:2, by vol.) solvent system (solvent I) and stained with benzidine-trichloroacetic acid reagent (Bacon & Edelman, 1951). The fractions were mixed according to their chromatographic behaviour. One fraction (2.3g.) containing a mixture of oligosaccharides with R_{lactose} values between 0.3 and 1.0 was further fractionated on a charcoal-Celite column (56 cm. $\times$ 2 cm.) developed with an aqueous ethanol gradient (0–15%, v/v, ethanol) maintained at pH 3

with formic acid (Taylor & Whelan, 1962). The fractions collected within the ethanol concentration range 5.6–8.5% (v/v) were neutralized by shaking with Permutit De-Acidite FF-IP (CO_3^{2-} form), evaporated under reduced pressure and dried from the frozen state. The mixed oligosaccharides obtained (333 mg.) were separated into two major fractions by preparative paper chromatography on Whatman no. 4 paper developed with solvent I. Component *a* (yield 32 mg.) had R_{lactose} 0.53 and component *b* (yield 43 mg.) had R_{lactose} 0.62.

From fraction *a* the trisaccharide *O*- β -D-galactopyranosyl-(1 $\rightarrow$ 4)-*O*-(*N*-acetyl-D-glucosaminopyranosyl)-(1 $\rightarrow$ 6)-D-galactose has already been identified (Aston *et al.* 1968). Fraction *b* was shown by electrophoresis at pH 10 in borate buffer to contain two components, with M_{Glc} 0.57 and 0.30. The material was dissolved in a small amount of aqueous 50% ethanol, methanol was added until the solution became cloudy and the mixture was kept at -10° for several days. Gelatinous particles formed and were recovered from the solution by decantation. The substance was washed with methanol, dissolved in water and dried from the frozen state. The material (12 mg.) now behaved as a single substance when examined by electrophoresis (M_{Glc} 0.57 in borate buffer, pH 10) and by chromatography on Whatman no. 1 paper [R_{lactose} 0.54 in solvent I; R_{lactose} 0.40 in ethyl acetate-pyridine-water (10:4:3, by vol.) (solvent II)].

Acid hydrolysis (0.5N-HCl for 19 hr. at 100°) of the substance gave two sugars only, galactose and glucosamine, when the hydrolysate was examined by paper chromatography. Galactose, determined by the method of Dubois, Gilles, Hamilton, Rebers & Smith (1956), and glucosamine, determined by the method of Rondle & Morgan (1955), were present to the extent of 55% and 28% respectively. The molar ratio of galactose to glucosamine was 1.94:1.00. Reduction of the substance with an excess of sodium borohydride at room temperature for 3 hr. halved the galactose content, whereas the glucosamine remained unchanged. The substance, $[\alpha]_D + 20^\circ$ (c 1 in water) was considered to be a trisaccharide with galactose at the reducing end.

Table 1. Oxidation of the trisaccharide with 25mM-sodium metaperiodate at 18–20°

	Oxidation time (hr.) ...	1	2	4	20	24	Theory
Moles of periodate reduced/mole of trisaccharide		3.66	—	4.02	—	3.84	4.00
Moles of formic acid formed/mole of trisaccharide		1.48	1.97	1.84	1.96	—	2.00
Moles of formaldehyde liberated/mole of trisaccharide		—	—	0.08	—	—	0.00
Moles of formaldehyde liberated/mole of trisaccharide after reduction		—	1.87	1.94	—	—	2.00

It gave a pink colour with alkaline triphenyltetrazolium chloride (Wallenfels, 1950; Feingold, Avigad & Hestrin, 1956; Côté & Morgan, 1956), indicating that there was no substituent on C-2 of the reducing sugar.

Hydrolysis of the substance with 2N-acetic acid at 100° overnight gave products on paper chromatography that were indistinguishable from (i) unchanged substance, (ii) lacto-*N*-biose I [*O*-β-D-galactopyranosyl-(1→3)-*N*-acetyl-D-glucosamine], (iii) galactose, (iv) *N*-acetylglucosamine and (v) a product with R_{lactose} 0.98 in solvent II. Products i, ii, iv and v gave a positive indirect Ehrlich reaction (Aminoff, Morgan & Watkins, 1952).

Treatment of the trisaccharide with 5% triethylamine in aqueous 50% methanol at 74° for 1 hr. brought about only slight degradation. After 4 hr. heating under the same conditions degradation was incomplete, and the stability observed was similar to that shown by the (1→4)- and (1→6)-linked disaccharides, lactose and melibiose, included in the test. One of the several degradation products obtained from the trisaccharide gave a positive direct Ehrlich reaction. This material was chromatographically identical, R_{lactose} 3.26 in solvent I, with a chromogen (Kuhn & Krüger, 1956, 1957) produced under the same conditions by the alkaline degradation of either lacto-*N*-biose I or *N*-acetylglucosamine.

The trisaccharide was completely hydrolysed in 4 hr. at 37° to galactose and a disaccharide, R_{lactose} 1.0 in solvent II, by a β-galactosidase preparation from *Trichomonas foetus*. The disaccharide was further hydrolysed to galactose and *N*-acetylglucosamine by a β-*N*-acetylglucosaminidase obtained from the same organism.

Methylation of the trisaccharide with methyl iodide in dimethylformamide and BaO-Ba(OH)₂·8H₂O followed by methanolysis with 0.5N-HCl in methanol at 80° gave a mixture of methylated glycosides that when examined by gas-liquid chromatography gave peaks with retention times identical with those given by authentic specimens of methyl 2,3,4,6-tetra-*O*-methylgalacto-

side and methyl 2,3,6-tri-*O*-methylgalactoside. A methylated derivative of glucosamine, 4,6-di-*O*-methylglucosamine, was identified after the mixture had been hydrolysed with 2N-HCl at 100° and examined on an amino acid analyser (Heyns & Kiessling, 1967).

The results obtained by oxidation of the trisaccharide with 25mM-sodium metaperiodate (Belcher, Dryhurst & MacDonald, 1965a,b) are given in Table 1. The values obtained for the reduction of periodate, the formic acid produced and the formaldehyde liberated, when taken together with the other evidence, are in agreement with those expected for the trisaccharide *O*-β-D-galactopyranosyl-(1→3)-*O*-(*N*-acetyl-β-D-glucosaminopyranosyl)-(1→4)-D-galactose.

In the human blood-group substances trisaccharides of the type galactose-*N*-acetylglucosamine-galactose occur in the carbohydrate chains with at least four different sequences of glycosidic bonds. In addition to the trisaccharides previously described having (1→3)-(1→3)-, (1→4)-(1→3)- and (1→4)-(1→6)-linkages the evidence now presented demonstrates a fourth sequence, (1→3)-(1→4), and thus further emphasizes the complexity of the carbohydrate moiety of the blood-group active substances.

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