TWO ADDITIONAL OLIGOSACCHARIDES OBTAINED FROM AN H-ACTIVE GLYCOPROTEIN BY ACID HYDROLYSIS

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With the recognition of many di- and trisaccharides as components of the carbohydrate chains in the blood-group specific glycoproteins, the elucidation of the number, sequence and anomeric form of the sugar units in the branched heterosaccharide chains that occur in the native macromolecule becomes of considerable importance. To obtain this information many more oligosaccharides larger than trisaccharides must be identified. Two additional fragments, a tetrasaccharide, β-Gal-(1→3)-β-GNAc-(1→4)-[Fuc-(1→6)-]Gal, and a hexasaccharide are described in this communication.

Experimental and Results. Group specific H substance (No. 511, 15 g.) (analytical figures: fucose 24%, reducing sugar 56%, hexosamines 23%, nitrogen 4.9% and amino acids 16.5%) was dissolved in polystyrene sulphonic acid (900 ml., 0.04 N, pH 2.1) and continuously hydrolysed and dialysed; the diffusate (10.4 g.) was fractionated on columns of Sephadex G-10 and charcoal-Celite (1:1) as already described (Aston et al, 1968a). The fractions obtained from the charcoal-Celite column within the concentration range 8.5 to 12.0% $^{v}/_{v}$ aqueous ethanol gave 325 mg. of material (Fraction A). After the ethanol gradient had reached 15% 1.5 litres of 20% ethanol were passed through the column and 250 mg. of oligosaccharides were obtained (Fraction B).

Fraction A was resolved further by chromatography on Whatman No.40 paper developed with ethyl acetate-pyridine-water, 2:1:2 by vol., upper phase (solvent a) to yield the tetrasaccharide β-Gal-(1→4)-β-GNAc-(1→6)-β-Gal-(1→3)-GNAc, $[\alpha]_D$ = -21.4° (c, 0.3), already described (Aston et al, 1968a). Another oligosaccharide (7.3 mg., $R_{lactose}$ 0.65, solvent a and 0.56 in ethyl acetate-pyridine-water, 10:4:3 by vol. (solvent b); M_G 0.38 in borate buffer pH 10) was also recovered. This substance gave galactose, glucosamine and fucose on complete acid hydrolysis (molar ratio 2.0:1.1:1.0 respectively). The galactose content of the substance after reduction with $NaBH_4$ was decreased by 46.5% indicating that the substance was a tetrasaccharide and that galactose was the reducing sugar residue.

The tetrasaccharide gave a weak indirect Ehrlich reaction (Aminoff et al, 1952) and a pink colour with alkaline triphenyltetrazolium chloride indicating there was no substituent on C2 of the sugar at the reducing end (Feingold et al, 1956).

Hydrolysis of the tetrasaccharide with 2 N acetic acid at 100° overnight gave products that on paper chromatography were indistinguishable from (i) unchanged substance, (ii) the trisaccharide β-Gal-(1→3)-β-GNAc-(1→4)-Gal (Aston et al, 1968b), (iii) lacto-N-biose I (β-Gal-(1→3)-GNAc), (iv) galactose, (v) N-acetylglucosamine, (vi) fucose and (vii) a product with $R_{lactose}$ 1.07 (solvent b). Products (i), (ii), (iii), (v) and (vii) gave an indirect Ehrlich reaction.

The tetrasaccharide on incubation with a β-galactosidase preparation obtained from T.foetus yielded galactose and a substance with $R_{lactose}$ 0.86 (solvent b). The tetrasaccharide had neither H nor Le^a specificity (Watkins and Morgan, 1962).

On oxidation with 0.02 M sodium metaperiodate (Belcher et al, 1965a and b) at pH 5 and room temperature the tetrasaccharide consumed 5.8 moles periodate (theory 6.0) after 4 hr., and 3.0 moles of formic acid (theory 3.0) were liberated. The glycitol of the tetrasaccharide obtained by reduction with $NaBH_4$ gave on oxidation 1.2 moles of formaldehyde after 2 hr. (theory 1.0).

After methylation and hydrolysis of the tetrasaccharide 2,3,4,6-tetra-O-methylgalactose, 2,3,4-tri-O-methylfucose and 4,6-di-O-methylglucosamine were identified chromatographically as previously described (Aston et al, in press).

Treatment of the tetrasaccharide with 5% triethylamine in aqueous 50% methanol at 75° for 4 hr. brought about a slow degradation at a rate similar to that observed for the trisaccharide β-Gal-(1→3)-GNAc-(1→4)-Gal under the same conditions. Galactose and "chromogens" (Kuhn and Krüger, 1956, 1957) were identified among the degradation products.

The results of methylation analysis and partial acid hydrolysis imply that the fucose is attached as a branched structure to the galactose at the reducing end of the tetrasaccharide. Periodate oxidation results indicated that this galactose residue was substituted in positions C4 and C6. No free fucose was detected in the alkaline degradation products, but the formation of galactose and a chromogen suggested that the Gal-(1→3)-GNAc unit was joined to the reducing galactose residue by a more alkali-labile linkage (i.e. (1-4)) than was fucose (see Whistler and BeMiller, 1958). The attachment of fucose to the C6 position would result in the formation of an alkali-stable compound with fucose attached to an unsaturated residue formed from galactose when the Gal-(1→3)-GNAc unit is eliminated from the C4 position.

The above evidence indicates that the new tetrasaccharide is β-Gal-(1→3)-β-GNAc-(1→4)-[Fuc-(1→6)-]Gal.

A larger sugar fragment was isolated from Fraction B by chromatography on Whatman No.40 paper developed by solvent a. The oligosaccharide (3.7 mg.) moved as a single component on paper chromatography ($R_{Isomaltopentaose}$ 0.97, solvent a and 0.85 solvent b) and on electrophoresis (M_G 0.30, borate buffer pH 10). Complete acid hydrolysis showed that the substance was composed of galactose and glucosamine in the molar ratio 1.00 : 1.09. After reduction of the oligosaccharide with $NaBH_4$ the galactose content remained unchanged, but 35% of the glucosamine was reduced to glucosaminitol. These results together with the chromatographic behaviour of the compound ($R_{lactose}$ 0.08, solvent b) suggested that the oligosaccharide was a hexasaccharide having glucosamine as the reducing sugar unit.

The hexasaccharide gave a strong indirect Ehrlich reaction. Degradation in the triethylamine reagent resulted in many products, including two substances $R_{lactose}$ 0.57 and 3.64 (solvent b) which gave a direct Ehrlich reaction. The latter chromogen was chromatographically identical with a chromogen formed by the degradation of lacto-*N*-biose I under the same conditions.

Among the partial acid hydrolysis (2 *N* acetic acid, 100°, 18 hr.) products of the hexasaccharide were substances chromatographically indistinguishable in solvents a and b from (i) unhydrolysed substance, (ii) the tetrasaccharide (β-Gal-(1→4)-β-GNAc-(1→6)-β-Gal-(1→3)-GNAc), (iii) the trisaccharide (β-Gal-(1→4)-β-GNAc-(1→6)-Gal), (iv) the disaccharide (β-GNAc-(1→6)-Gal), (v) lacto-*N*-biose I, (vi) galactose and (vii) *N*-acetylglucosamine (for (ii), (iii) and (iv) see Aston *et al*, 1968a). The products (i), (ii), (v) and (vii) all gave an indirect Ehrlich reaction.

The β-galactosidase preparation converted the hexasaccharide into two substances, galactose and a product $R_{lactose}$ 0.18 (solvent *b*).

Methylation of the hexasaccharide followed by hydrolysis yielded products which were identified as 2,3,4,6-tetra-*O*-methylgalactose, 2,3,4-tri-*O*-methylgalactose, 2,4,6-tri-*O*-methylgalactose, 3,6-di-*O*-methylglucosamine and 4,6-di-*O*-methylglucosamine. The ratio of the 4,6- to the 3,6- glucosamine derivatives was estimated to be 1.3 : 1.0, a ratio which is considered to indicate a ratio of 2:1 for the C3 and C4 substituted hexosamines in the hexasaccharide, since the C3 linked glucosamine at the reducing end of the chain would be partially degraded under the alkaline conditions of methylation.

These results imply that the hexasaccharide is an unbranched structure with a galactose residue at the non-reducing end of the chain. The two other galactose residues are substituted at positions 3 and 6 respectively. Two of the hexosamines in the hexasaccharide are apparently substituted at C3 and the third molecule of hexosamine at C4. The results of partial acid hydrolysis suggest that the tetrasaccharide β-Gal-(1→4)-β-GNAc-(1→6)-β-Gal-(1→3)-GNAc is part of the hexasaccharide molecule. Therefore three possible structures may be proposed for the hexasaccharide:

(i) β-Gal-(1→4)-β-GNAc-(1→6)-β-Gal-(1→3)-GNAc-(1→3)-Gal-(1→3)-GNAc

(ii) β-Gal-(1→3)-GNAc-(1→3)-β-Gal-(1→4)-β-GNAc-(1→6)-β-Gal-(1→3)-GNAc

(iii) β-Gal-(1→3)-β-Gal-(1→4)-β-GNAc-(1→6)-β-Gal-(1→3)-GNAc-(1→3)-GNAc

Lack of material prevented further evidence being obtained to differentiate between these structures. Structure (i) is favoured since of the three it best explains the extent and ease with which degradation occurs when the hexasaccharide is treated with alkali.

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