OLIGOSACCHARIDES CONTAINING A (1-6) GLYCOSIDIC LINKAGE OBTAINED FROM HUMAN BLOOD-GROUP SPECIFIC GLYCOPROTEINS

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A, B, H, Le^a and Le^b blood-group active glycoproteins from human secretions and tissue fluids yield many di- and oligosaccharides on partial acid hydrolysis, alkaline degradation and hydrazinolysis. The evidence indicates that in the many carbohydrate chains in each specific substance there is a common pattern of alternate galactose and amino sugar units, but the nature of the glycosidic linkages between the different sugars is not always the same. It is firmly established that 1→3 and 1→4 linkages occur (see Watkins, 1966), and there is now evidence (Yosizawa, 1961. Lloyd and Kabat, 1967) for 1-6 linkages. In this communication the isolation from group specific glycoproteins of a disaccharide, a trisaccharide and a tetrasaccharide each containing a (1→6) linkage is described.

Experimental and Results. Group specific H substance (15 g.) from a single specimen of ovarian cyst fluid was dissolved in 900 ml. of 0.04 N in-diffusible, water-soluble, polystyrene sulphonic acid (Painter and Morgan, 1961), and continuously hydrolysed and dialysed until the formation of diffusible fragments had almost ceased. The diffusate was concentrated, dried from the frozen state (10.4 g.) and chromatographed in portions on a column (82 x 3.5 cm.) of Sephadex G-15. The fractions were mixed according to their chromatographic behaviour. Material (2.3 g.) with $R_{lactose}$

values between 0.3 and 1.0 in ethyl acetate-pyridine-water (10:4:3) (solvent a) was further fractionated on a column (56 x 2 cm.) of charcoal-Celite, using gradient elution with aqueous ethanol (0 - 15% ethanol) maintained at pH 3 with formic acid. The fractions obtained between 5.6 and 8.5%, and 8.5 and 12.0% ethanol were separately neutralized with Permutit De-Acidite FF-IP in its carbonate form. Oligosaccharide I (32 mg.), the major component in the material eluted between 5.6 and 8.5% $^{v}/_{v}$ ethanol, was obtained as a chromatographically ($R_{lactose}$ 0.34, solvent a and 0.52 in ethyl acetate-pyridine-water 2:1:2, solvent b) and electrophoretically (M_G 0.60 in borate buffer, pH 10) homogeneous substance after purification by preparative paper chromatography (solvent b) and gel filtration through Sephadex G-15. On acid hydrolysis Oligosaccharide I gave galactose and glucosamine in the molar ratio of 1.94 : 1.00 respectively, and after reduction with sodium borohydride ($NaBH_4$) the galactose content was only half of the original value while the amount of hexosamine was unchanged. These findings, together with the chromatographic behaviour of Oligosaccharide I suggested it was a trisaccharide having galactose at the reducing end. The trisaccharide was slowly degraded in 2.5% triethylamine in 50% aqueous methanol at 80° (Rege, Painter, Watkins and Morgan, 1964) and gave a small amount of galactose and a disaccharide which gave no colour with Ehrlich's reagent and was chromatographically indistinguishable from N-acetyl-lactosamine (O-β-D-galactosyl-(1→4)-N-acetyl-D-glucosamine). Partial hydrolysis of the trisaccharide with 2-N-acetic acid at 100° for 17 hr. gave N-acetyl-lactosamine, galactose and N-acetylglucosamine. On oxidation of the trisaccharide with 0.012 M sodium metaperiodate ($NaIO_4$) at room temperature 5.8 moles periodate (theory 5.0) were consumed after

4 hr., and 3.2 moles of formic acid (theory 3.0) and 0.28 moles of formaldehyde (theory 0.0) were liberated. The glycitol of the trisaccharide obtained by reduction with $NaBH_4$ gave on oxidation 1.22 moles of formaldehyde after 4 hr. (theory 1.0).

The trisaccharide was methylated and hydrolysed as previously described (Marr, Donald, Watkins and Morgan, 1967) and the methyl 2,3,4,6 tetra-O-methylgalactosides and methyl 2,3,4 tri-O-methylgalactosides were identified by gas-liquid chromatography. 3,6 di-O-methylglucosamine was identified on an amino acid analyser. On the basis of the above evidence the trisaccharide is believed to be O-β-D-galactosyl-(1→4)-O-(N-acetyl-D-glucosaminyl)-(1→6)-D-galactose.

Oligosaccharide II (15 mg.) obtained from the 8.5 to 12% ethanol effluent from the charcoal column and purified by procedures similar to those used for the trisaccharide was chromatographically ($R_{lactose}$ 0.29, solvent a, and 0.48 solvent b) and electrophoretically (M_G 0.35 in borate buffer pH 10) homogeneous, and gave only galactose and glucosamine, in equimolar ratio (1.02 : 1.00), on complete acid hydrolysis. The chromatographic behaviour of Oligosaccharide II indicated it was a tetrasaccharide. After reduction with $NaBH_4$ and hydrolysis the galactose content did not change, but glucosamine was reduced to about half, indicating that this sugar was at the reducing end of the molecule. The molar ratio of the glucosamine to glucosaminitol in the hydrolysis products was 1.0 : 1.09. The tetrasaccharide gave a strong Ehrlich reaction after treatment with mild alkali, showing that the N-acetylglucosamine at the reducing end was unsubstituted at the C4 position. The oligosaccharide was degraded in aqueous methanolic triethylamine at 85° at a rate similar to that of lacto-N-biose I (O-β-D-

galactosyl-(1→3)-N-acetyl-D-glucosamine) and the degradation products contained a substance chromatographically and electrophoretically indistinguishable from the trisaccharide (Oligosaccharide I) and a "fast-running" compound, $R_{lactose}$ 6.4 (solvent a), which gave an immediate, positive Ehrlich reaction with p-dimethylaminobenzaldehyde similar to the product formed by lacto-N-biose I degraded under the same conditions. Partial acid hydrolysis of the tetrasaccharide with 2N-acetic acid at 100° for 17 hr. gave substances chromatographically indistinguishable from Oligosaccharide I, N-acetyl-lactosamine, lacto-N-biose I, galactose and N-acetylglucosamine.

On methylation analysis Oligosaccharide II gave 4,6 di-O-methylglucosamine in addition to the methyl ethers obtained from the trisaccharide. The evidence therefore indicates that the tetrasaccharide is O-β-D-galactosyl-(1→4)-O-(N-acetyl-D-glucosaminyl)-(1→6)-O-β-D-galactosyl-(1→3)-N-acetylglucosamine.

Group A substance (9 g.) of human origin was subjected to hydrazinolysis using hydrazine sulphate as catalyst (Yosizawa, Sato and Schmid, 1966). The product was dried in vacuo, the residue dissolved in water, the relatively large carbohydrate fragments separated from amino acids by gel filtration through Sephadex G-15, and the oligosaccharides (4.6 g.) hydrolysed with 0.5 N H_2SO_4 at 100° for 5 hr. The glycosidic bonds next to the free NH_2 groups, formed from the acetamido groups in the amino sugars during hydrazinolysis, are largely resistant to cleavage because of the electrostatic shielding effect of the $-NH_3^+$ group (Moggridge and Neuberger, 1938) and in consequence a number of oligosaccharides having amino sugar at the non-reducing ends are obtained. A solution of the oligosaccharides in 0.33 N HCl was passed through a column of Amberlite CG.120 resin and eluted with

0.33 N HCl. From the eluate a substance was recovered that, after reacetylation (Roseman and Ludowieg, 1954) and separation from other fragments by chromatography on Whatman No.40 paper using solvent a, had $[\alpha]_D$ + 10° (c,1), $R_{lactose}$ 0.89 (solvent a) and M_G 0.68 (borate buffer, pH 10). The substance contained equimolar amounts of glucosamine and galactose, and after reduction with $NaBH_4$ and hydrolysis with acid gave glucosamine on analysis but no galactose. A positive reaction with triphenyltetrazolium chloride indicated that position C2 on the sugar at the reducing end of the substance was unsubstituted. The indirect Ehrlich reaction was negative and treatment of the substance with 2.5% triethylamine in 50% aqueous methanol at 80° gave little decomposition. On oxidation with 0.012 M $NaIO_4$ the substance consumed 4 moles of oxidant, 2 moles of formic acid were formed but no formaldehyde. Oxidation of the substance with $NaIO_4$ after reduction with sodium borohydride gave one mole of formaldehyde per mole of compound. After methylation of the substance and methanolysis, gas-liquid chromatography of the products revealed peaks identical with those given by the α and β forms of methyl 2,3,4-tri-O-methylgalactoside. On the basis of these observations the substance is the disaccharide, O-β-(N-acetyl-D-glucosaminyl)-(1→6)-D-galactose, first synthesized by Kuhn and Kirschenlohr (1954) and later isolated from hog gastric mucin A substance by Yosizawa (1961).

Summary. A disaccharide, O-β-(N-acetyl-D-glucosaminyl)-(1→6)-D-galactose, and two oligosaccharides, O-β-D-galactosyl-(1→4)-O-(N-acetyl-D-glucosaminyl)-(1→6)-D-galactose and O-β-D-galactosyl-(1→4)-O-(N-acetyl-D-glucosaminyl)-(1-6)-O-β-D-galactosyl-(1→3)-N-acetyl-D-glucosamine, have been obtained from blood-group specific glycoproteins.

The occurrence of the N-acetylglucosamine unit at the

reducing end of the tetrasaccharide gives evidence for the presence of at least two molecules of glucosamine in a single chain. The recognition of this additional glucosamine unit extends our knowledge of the sugar sequence in the chains and goes some way towards understanding the values found for the ratio of galactosamine:glucosamine in blood-group specific glycoproteins (see Watkins, 1966).

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