Short Communications

Two New Oligosaccharides Obtained from an Le^a -Active Glycoprotein

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(Received 10 October 1968)

The results of a further examination of the products of alkaline degradation of an Le^a -blood-group-specific glycoprotein obtained from an ovarian-cyst fluid (Rege, Painter, Watkins & Morgan, 1964) have revealed the presence of two new oligosaccharides; their structure is based on the evidence given in this paper.

A preparation of Le^a -active glycoprotein [no. 579, 21g.; analytical values: fucose 2.9%, galactose 30.4%, *N*-acetylhexosamine 32.2% (molar ratio galactosamine:glucosamine 1:1.56) and amino acids 28%] was dissolved in 1100 ml. of 0.044 M-polyvinylbenzyltriethylammonium carbonate, pH 8.6, and continuously degraded and dialysed, as described by Marr, Donald, Watkins & Morgan (1967).

The diffusible material (9g.) was fractionated on a column (84 cm. $\times$ 3.5 cm.) of Sephadex G-15 with water as eluent. The fractions were examined by paper chromatography (Whatman no. 40 paper was used throughout the experimental work) with ethyl acetate-pyridine-water (10:4:3, by vol.) (solvent *a*) and pooled on the basis of their chromatographic behaviour. Sugar fragments having R_{lactose} values between 0.09 and 1.1 were recovered (3.2g.) and fractionated further on a column (51 cm. $\times$ 2.5 cm.) of charcoal-Celite (1:1, w/w) by using stepwise elution with aqueous-ethanol mixtures. The material (210mg.) obtained from the first litre of aq. 5% (v/v) ethanol was further fractionated by gel filtration on columns (150 cm. $\times$ 0.8 cm.) of Sephadex G-15 and Bio-Gel P-2 and by repeated preparative paper chromatography in ethyl acetate-pyridine-water (2:1:2, by vol.; upper phase) (solvent *b*). The disaccharides 4-*O*- β -D-galactosyl-*N*-acetyl-D-glucosamine (*N*-acetyl-lactosamine) and 3-*O*- β -D-galactosyl-*N*-acetyl-D-galactosamine were identified (Painter, Rege & Morgan, 1963).

A third component (4.3mg.) was also isolated which was chromatographically pure (R_{lactose} 0.24 and 0.33 in solvents *a* and *b*); it was electrophoretically homogeneous, M_{Glc} 0.45, on paper in borate buffer, pH 10. Nevertheless the substance was contaminated with non-carbohydrate material, as shown by the analytical values determined for the constituent sugars, which were fucose (11.8%),

galactose (25.6%) and *N*-acetylglucosamine (13.3%). The molar proportions 1.00:1.97:0.84 for the three sugars indicated that the oligosaccharide component in the material was a tetrasaccharide. The substance had $[\alpha]_{\text{D}} -32^\circ$ (*c* 0.5 in water); this value is corrected for inert material, which is assumed to be optically inactive. After reduction with NaBH_4 52% of the galactose was destroyed, whereas the amounts of the other two sugars remained unchanged.

The products of partial acid hydrolysis (2*N*-acetic acid for 18 hr. at 100°) of the tetrasaccharide contained components chromatographically and electrophoretically identical with the constituent monosaccharides, the disaccharides *N*-acetyl-lactosamine and 3-*O*- β -(*N*-acetyl-D-glucosaminyl)-D-galactose (lacto-*N*-biose II) and the trisaccharide *O*- β -D-galactosyl-(1 $\rightarrow$ 4)-*O*- β -(*N*-acetyl-D-glucosaminyl)-(1 $\rightarrow$ 3)-D-galactose (Rege, Painter, Watkins & Morgan, 1963).

When the tetrasaccharide was subjected to partial alkaline degradation [5% (v/v) triethylamine in aq. 50% (v/v) methanol at 75° for 1 hr. and 4 hr.] it was almost completely degraded at a rate similar to that found for lacto-*N*-biose II. Disaccharides having reducing terminal hexoses linked (1 $\rightarrow$ 2), (1 $\rightarrow$ 4) and (1 $\rightarrow$ 6) when treated under identical conditions were either unchanged or only partly degraded. Among the degradation products of the tetrasaccharide, none of which gave a direct Ehrlich reaction, fucose and a much smaller quantity of galactose were recognized.

Methylation (Kuhn, Baer & Seeliger, 1958; Kuhn & Trischmann, 1961) of the tetrasaccharide followed by acid hydrolysis gave 2,3,4,6-tetra-*O*-methylgalactose, 2,4,6-tri-*O*-methylgalactose, 2,3,4-tri-*O*-methylfucose and 6-*O*-methylglucosamine, which were identified chromatographically (see Aston, Donald & Morgan, 1968). It is concluded that the tetrasaccharide, which has no Le^a activity, is *O*- β -D-galactosyl-(1 $\rightarrow$ 4)-[*O*-L-fucosyl-(1 $\rightarrow$ 3)]-*O*- β -(*N*-acetyl-D-glucosaminyl)-(1 $\rightarrow$ 3)-D-galactose (I).

The material recovered from the subsequent fractions eluted from the charcoal-Celite column with aq. 5% (v/v) ethanol (9l.) was repeatedly chromatographed on a Bio-Gel P-2 column

(I)

(II)

(150 cm. $\times$ 0.8 cm.), and one fraction (72 mg.) was further purified by preparative paper chromatography (solvent *b*) and gave a substance (21 mg.) that appeared to be homogeneous and was then crystallized from aqueous methanol.

The crystalline substance, m.p. 214–217°, $[\alpha]_D^{+14}$ (c 0.5 in water) was chromatographically (R_{lactose} 0.47 and 0.67 in solvents *a* and *b* respectively) and electrophoretically (M_{Glc} 0.30 in borate buffer, pH 10) homogeneous. Analytical values were galactose 28%, *N*-acetylglucosamine 32% and *N*-acetylgalactosamine 31%, indicating the molar proportions 1:1:1.0:1.0 respectively. After reduction of the substance with NaBH_4 the *N*-acetylgalactosamine was completely converted into *N*-acetylgalactosaminitol, determined as described by Donald (1968). Galactose and *N*-acetylglucosamine were not reduced. The compound appeared to be a trisaccharide with *N*-acetylgalactosamine at the reducing end.

Methylation and hydrolysis of the trisaccharide, by using the same procedure as for the tetrasaccharide, gave 2,3,4,6-tetra-*O*-methylgalactose and 3,6-di-*O*-methylglucosamine. Methylated galactosamine standards were not available.

Oxidation (Belcher, Dryhurst & MacDonald, 1965*a,b*) of the crystalline trisaccharide with 0.025M NaIO_4 at 18–20°, both before and after reduction with NaBH_4 , gave approx. 0.1 mole of

formaldehyde/mole of sample, indicating that position 6 of the *N*-acetylgalactosamine was substituted (theoretical yield of formaldehyde 0.0).

The trisaccharide gave a weak indirect Ehrlich reaction, similar to that given by 6-*O*- β -D-galactosyl-*N*-acetyl-D-glucosamine. Partial acid hydrolysis of the trisaccharide with 2*N*-acetic acid at 100° for 4 hr. gave products chromatographically identical with *N*-acetylglucosamine, *N*-acetylgalactosamine, galactose and *N*-acetyl-lactosamine. Also present was a component, R_{lactose} 1.13 (solvent *a*), that gave a weak indirect Ehrlich reaction. A substance chromatographically identical with this and presumably a disaccharide composed of *N*-acetylglucosamine and *N*-acetylgalactosamine was observed, together with galactose, when the trisaccharide was incubated with a β -galactosidase from *Trichomonas foetus*.

On the basis of the evidence given the crystalline trisaccharide is believed to be *O*- β -D-galactosyl-(1 $\rightarrow$ 4)-*O*-(*N*-acetyl-D-glucosaminyl)-(1 $\rightarrow$ 6)-*N*-acetyl-D-galactosamine (II).

The proposed structure is supported by the results of partial alkaline degradation. Treated with 5% triethylamine in aq. 50% methanol for 4 hr. at 75° the trisaccharide was degraded at a rate comparable with that of 6-*O*- β -D-galactosyl-*N*-acetyl-D-glucosamine, whereas the corresponding

(1→3)-linked disaccharide was largely destroyed and the (1→4)-linked compound was unchanged when heated under similar conditions. *N*-Acetyl-lactosamine was identified among the degradation products of the trisaccharide, together with a substance, R_{lactose} 1.02 (solvent *a*), that gave an immediate and strong direct Ehrlich reaction.

A material with similar properties and giving a direct Ehrlich reaction was obtained from the 15%-ethanol eluate from the charcoal-Celite column and further purified by gel filtration on a column of Bio-Gel P-2 and preparative paper chromatography (solvent *b*). The compound (28 mg.) had R_{lactose} 1.02 and 1.17 (solvents *a* and *b*), M_{Glc} 0.15 in borate buffer, pH 10, and gave a direct Ehrlich reaction, indicating the presence of a chromogen derived from an *N*-acetyl-amino sugar (see Neuberger, Marshall & Gottschalk, 1966). Analysis showed the presence of galactose (25.8%) and *N*-acetylglucosamine (25.5%). The sugars were present in the molar ratio 1.2:1.0 respectively. When the substance was subjected to partial acid hydrolysis or partial alkaline degradation, under the conditions described for the trisaccharide, *N*-acetyl-lactosamine was identified. Further, galactose was released when the compound was treated with the β -galactosidase obtained from *T. foetus*. The chromogenic substance is present in considerable quantity in the products of the alkaline degradation of the blood-group substances. The structural relationship between this chromogenic compound and the crystalline trisaccharide was clarified by the identification of 2,3,4,6-tetra-*O*-methyl-galactose and 3,6-di-*O*-methylglucosamine in the products of methylation and hydrolysis of both compounds. Such stable chromogenic structures are known to be formed by the action of alkali on oligosaccharides containing a 6-substituted *N*-acetylhexosamine at the reducing end (Neuberger *et al.* 1966).

From the evidence given the chromogenic compound is believed to be *O*- β -D-galactosyl-(1→4)-*N*-acetyl-D-glucosaminyl-(1→6)-R, where R is a chromogenic structure.

The trisaccharide and the chromogen, at a dilution of 1:1600, inhibited the precipitation reaction between Le^a blood-group substance (diluted 1:10000) and undiluted horse anti-(type XIV pneumococcal) serum. *N*-Acetyl-lactosamine gave comparable inhibition on a weight basis, whereas *O*- β -D-galactosyl-(1→3)-*N*-acetyl-D-glucosamine was virtually inactive in the test system. This finding further supports the conclusion that *N*-acetyl-lactosamine is the disaccharide unit at the non-reducing end of the trisaccharide and of the chromogenic substance derived from it.

The β -galactosidase preparation was kindly supplied by Professor Winifred M. Watkins. The work was supported by a grant from the Medical Research Council.

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