SOME PRODUCTS OF THE DEGRADATION OF BLOOD GROUP SUBSTANCES BY ALKALINE BOROHYDRIDE *

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Alkaline borohydride produces fragments from blood group substances, some of which (A_3, B_3, H_4) appear to contain the entire antigenic determinants (Schiffman et al 1964 a). From the immunochemical and analytical properties of these partially purified fragments possible structures for these determinants were suggested (Schiffman et al 1964 b). For example, A_3 was thought to be a hexa or heptasaccharide consisting of 1 mole each of N-acetyl-D-galactosamine, D-galactose, N-acetyl-D-glucosamine, 2 moles of L-fucose, and a reduced end group. Some of these fractions have now been further purified and the action of alkaline borohydride on hog mucin (A+H) substance studied. A fucose containing oligosaccharide was obtained from A substance by Morgan (1962).

Materials and methods were described previously (Schiffman et al 1964 a,b; Kabat 1961). Fragments isolated from human ovarian cyst fluid MSS (A) by preparative paper chromatography using propanol-ethyl acetate-water (6:1:3) were further purified on paper in n-butanol-pyridine-water (6:4:3) (solvent 1) or 6:1:1 (solvent 2). Solvent 1 was used for fragments having an R_f less than lactose and solvent 2 for those with greater R_f. Fragments from ovarian cyst fluid MSM (A) and hog mucin (A+H) substances were isolated by repeated paper chromatography in either solvent 1 or 2 only. All fractions were further purified by chromatography on Darco G-60 charcoal-celite columns using ethanol gradients for elution

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Table 1
ANALYTICAL PROPERTIES OF ISOLATED OLIGOSACCHARIDES

Compound	R lac [1]	% Fucose	% Galactose	% Hexosamine	% N-Ac-hexosamine	% N	NAc-hexosamine / Hexosamine [2]	Molar Ratios: Fucose	Galactose	Hexosamine	N	$[\alpha]_{5461}^{24^\circ}$
MSM A_3	0.52	19.0	25.3	37.0	29.0	3.4	0.64	1.0	1.18	1.75	2.0	+ 27.0°
MSS A_3	0.52	17.0	19.0	32.0	24.0	2.8	0.66 [3]	1.0	1.0	1.70	1.9	+ 28.7°
Hog A_3	0.52	18.3	23.8	36.0	30.2	3.5	0.66	1.0	1.17	1.85	2.2	+ 25.8°
Theory		19.0	20.6	41.7		3.3	0.65	1.0	1.0	2.0	2.0	
MSM A_4a	0.96	28.0	1.3	26.0	11	2.7	0.35	1.0		0.86	1.1	+ 52°
Theory		30.9	0	33.9		2.6	0.31	1.0		1.0	1.0	
MSM A_4b [4]	0.96	2.3	24.7	19.2	25.7	2.6	1.08		1.0	0.78	1.35	
Theory		0	35.0	35.0		2.7	1.0		1.0	1.0	1.0	
MSM / Hog Fucosyl-galactitol	R_{gal} [5] 0.70	40.0	0	3.1	2.9	1.4						- 149°
	0.70	40.3				0.89						- 147°
Theory		50.0	0	0	0	0						
Hog G NAc-R	0.85	1.6	7.2 [6]	40.2	50.7	4.1	1.03			0.76	1.0	- 9.5°
Theory		0	0	51.0		4.0	1.0			1.0	1.0	

1) Solvent 1 2) GalNAc gives only 31% of the color of GNAc 3) The ratio of GN/GalN in an amino acid analyzer after hydrolysis in 2 N HCl for 2 hr. was 0.94 (by Dr. E. W. Bassett and Mrs. K. Pryzwansky) 4) Only 3 mg. of this fraction probably containing a large proportion of inert material was isolated 5) Solvent 2 6) R gives some color in the orcinol determination

(Whistler and Durso 1950).

A_3 fractions of comparable activity were isolated from all three sources. N-acetyl-D-galactosamine, L-fucose, D-galactose and N-acetyl-D-glucosamine were present in almost equimolar amounts (Table 1). The six moles of contaminating amino acids previously present in MSS A_3 were eliminated. Schiffman et al (1964 a,b) have suggested that MSS A_3 contained 2 moles of L-fucose but calculation of their data on a percentage basis shows that this is unlikely. They were also misled by the low value for galactose given by the Dische method (Dische 1955). In this study galactose was determined by the orcinol method (Winzler 1955) and corrected for fucose present (Nolan and Smith 1963). The newer data indicate that A_3 is a reduced pentasaccharide having the structure:

$$\begin{array}{l} \qquad\qquad\qquad\quad \alpha\text{-L-Fuc 1} \\ \qquad\qquad\qquad\quad \downarrow \\ \qquad\qquad\qquad\quad 2 \\ \alpha\text{-D-GalNAc-(1}\rightarrow\text{3)-}\beta\text{-D-Gal-(1}\rightarrow\text{ 3 or 4)-D-GNAc-R} \end{array}$$

Since it is nonreducing it must be terminated by a reduced sugar or sugar fragment (R). MSM A_3 consumed 3.92 moles periodate producing 0.92 mole formaldehyde in 7 hr. and 0.4 mole formic acid in 100 hr. per mole compound. Hydrolysis and chromatography (solvent 1) of the periodate oxidized products showed galactose and glucosamine. A_3 from MSS and from hog mucin gave similar results. The formaldehyde is evidently produced from the residue R. Since Rege et al (1963) have isolated O-β-D-galactopyranosyl-(1$\rightarrow$3)-O-(N-acetyl-β-D-glucosamino-pyranosyl)-(1$\rightarrow$3)-D-galactose from human A substance it would be expected that A_3 would be terminated by galactitol. However no galactitol could be detected by hydrolysis and chromatography in n-butanol-acetic acid-water (5:1:4) (solvent 3). Treatment of A_3 with alkaline permanganate indicated that R was unsaturated. From the formaldehyde released a molecular weight of about 900 (and about 150

for R) is obtained confirming that A_3 is a reduced pentasaccharide.

The A_4 fraction (85 mg) from MSM A substance was resolved by charcoal column chromatography into A_{4a} (51 mg) eluted with 15% ethanol and A_{4b} (23 mg) eluted with 18% ethanol. These were further purified on paper (solvent 1). Analysis of A_{4a} showed equimolar amounts of N-acetyl-D-galactosamine and L-fucose and hydrolysis and chromatography (solvent 3) demonstrated galactitol as well. The fragment shows somewhat more A activity than does N-acetyl-D-galactosamine. The structure proposed for A_{4a} is:

$$\begin{array}{l} \qquad\qquad\qquad\qquad\quad \alpha\text{-L-Fuc 1} \\ \qquad\qquad\qquad\qquad\qquad\quad \downarrow \\ \qquad\qquad\qquad\qquad\qquad\quad 2 \\ \alpha\text{-D-GalNAc-(1}\rightarrow 3)\text{-D-Galol} \end{array}$$

Per mole it consumed 4.98 moles of periodate in 6 hr., gave 1.1 moles of formaldehyde in 7 hr. and 1.4 moles formic acid in 10 hr. (Theory: 5, 1 and 2 moles respectively). The periodate data are also consistent with a 4,6 substituted galactitol but this latter structure is unlikely since the A active disaccharide 3-O-(N-acetyl-α-D-galactosaminopyranosyl)-D-galactose has been isolated from human blood group A substance (Côté and Morgan 1956, Schiffman et al 1961).

Unlike A_{4a}, A_{4b} rapidly decolorizes alkaline permanganate. Analysis and chromatography showed D-galactose and N-acetyl-D-glucosamine in equimolar amounts. A_{4b} consumed 3.08 moles of periodate giving 0.97 mole of formaldehyde in 6 hr. but no formic acid. After oxidation only glucosamine could be detected by hydrolysis and chromatography (solvent 1) showing the sequence of sugars and suggesting the structure:

$$\beta\text{-D-Gal-(1}\rightarrow 3 \text{ or } 4)\text{-D-GNAc-R}$$

From MSS, MSM and hog blood group substances a fraction consisting only of L-fucose and galactitol (by analysis and chromatography) was isolated. The compound consumed 4.94 moles of periodate in 6 hr. giving 1.1 moles of formaldehyde in 7 hr. and 1.6 moles of formic acid in 28 hr. The data is most consistent with the

structure 2-O-α-L-fucopyranosyl-D-galactitol (Theory: 5, 1 and 3 moles respectively) although the formic acid value is unexpectedly low. The α-linkage is indicated by the high negative rotation. Acetylation of the fraction from hog blood group substance using acetic anhydride in pyridine gave a crystalline acetate (m. p. 60-62°). 2-O-α-L-fucopyranosyl-D-galactose has recently been isolated from human H substance (Watkins and Morgan 1964) and a sample provided by Professor W.T.J. Morgan on reduction with sodium borohydride was chromatographically identical with our fucosyl-galactitol in solvents 1 and 2.

From hog mucin a fraction was isolated containing only D-glucosamine and the residue R. Per mole it consumed 1.84 moles of periodate and liberated 0.93 mole formaldehyde but no formic acid in 7 hr. From the formaldehyde a value of 150 for the molecular weight of R is again obtained. The structure proposed is O-(N-acetyl-β-D-glucosaminopyranosyl)-R.

Many other fragments are produced during the degradation but only two have been identified. From both MSM and hog mucin, crystalline galactitol (m.p. 187-188°; mixed m.p. 187-188°) has been isolated by preparative paper chromatography. Crystalline N-acetyl-D-galactosaminitol (m.p. 170-2°, mixed m.p. 170-2°) has been isolated from hog mucin.

Schiffman et al (1964 a,b) leave open the question of the mechanism of the action of alkali. From the series of compounds described here it is evident that although the initial cleavage may be of an especially labile bond, the main action of alkali is to degrade from the reducing end. It is evident that the borohydride does not completely protect liberated reducing groups from further attack by alkali. The formation of R in some fragments as a reduced unsaturated end group rather than of galactitol is unexpected. However it will be noticed that all fragments terminated by galactitol are substituted on its C_2, suggesting that such substitution prevents degradation of terminal galactose by alkali. Similarly, Kuhn

et al (1956) isolated 2-O-α-L-fucopyranosyl-D-galactose by degrading fucosidolactose and lacto-N-fucopentaose 1 with Na_2CO_3 at 100°. R could also be formed by other mechanisms, for instance, the alkaline elimination from galactose of a sugar or amino acid moiety. A 2,3 unsaturated sugar has been formed by the action of alkali on 2,3-di-O-methyl-D-glucose (Kenner and Richards 1956).

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