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STUDIES OF POLYSACCHARIDE STRUCTURE

PART I. A MODIFICATION OF THE SMITH DEGRADATION

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ABSTRACT

The diagnostic value is considered of degradation products arising when the Smith degradation is modified by the introduction of a methylation stage after borohydride reduction of the periodate-oxidised polysaccharide. The bond types occurring in two clinical dextrans were examined by conventional periodate oxidation and methylation analysis, and by the modified Smith-degradation.

INTRODUCTION

Methylation analysis is the most widely applied method for studying bond types occurring in polysaccharides. The Smith degradation[1–4] is also useful, and a variation of this method which includes an additional stage, namely, methylation of the product obtained by treatment of a polysaccharide in sequence with periodate and borohydride, and which was suggested by Smith and co-workers[1–4] for glycans containing (1→6)-branches, has been shown to be an efficient method of general applicability by Nánási and Lipták[5]. In comparison with the classical methylation procedure, an advantage of the modified Smith-degradation is the smaller number of reference compounds and the greater diagnostic value of the degradation products. Furthermore, there are greater differences in molecular weight and polarity of the degradation products associated with the modified Smith-method than those obtained after conventional methylation, and their separation can therefore be more easily achieved. By using this method, a complete analysis of bond types can be performed in contrast to the limited information obtainable by the original Smith-degradation.

RESULTS AND DISCUSSION

The compounds obtained by conventional Smith-degradation of polysaccharides composed of hexoses can be divided into two groups: carbonyl compounds (glycolaldehyde, D- or L-glyceraldehyde, hexose) and alditols (glycerol, erythritol or threitol).

For the Smith degradation modified by methylation[5], the diagnostic value of the degradation products is changed for the hexoses. Intact monosaccharide, when

released in the original Smith-degradation, simply reflects periodate-resistant monosaccharide residues. However, the method modified by methylation can yield mono-*O*-methylhexoses, di-*O*-methylhexoses (2,4-, 2,6-, 3,6-, and 4,6-) *containing no vicinal methoxyl groups*, and 2,4,6-tri-*O*-methylhexose, according to the linkage pattern of the periodate-resistant hexose: -3H*p*1- → 2,4,6-tri-*O*-methylhexose, -6H*p*1- → 2,4-di-*O*-methylhexose, -4H*p*1- → 3,6-di-*O*-methylhexose, -3H*p*1- → 4,6-di-*O*-methylhexose, -4H*p*1- → 2,6-di-*O*-methylhexose. The extrapolation of this scheme to other sugar types, *e.g.* 6-deoxyhexoses, is readily envisaged.

Incomplete periodate oxidation in the conventional Smith-degradation will result in the release of intact hexose additional to that deriving from periodate-resistant units. However, in the modified Smith-degradation, hexose units surviving because of incomplete oxidation will be converted into di- or tri-methyl ethers *containing vicinal methoxyl groups*. Thus, incomplete oxidation can thereby be detected.

The diagnostic value of alditols is also modified by supplementary methylation. Methylated derivatives of glycerol and tetritols will arise as follows: H*p*1- → 1,3-di-*O*-methylglycerol, -6H*p*1- → 1-*O*-methylglycerol, -4H*p*1- → 1,4-di-*O*-methylerythritol (threitol), -4H*p*1- → 1-*O*-methylerythritol (threitol). Thus, further data on linkage positions can be obtained from the alditols.

When secondary hydroxyl groups are involved at branch points, the associated carbon atom is retained in the degradation fragments and its configuration will be reflected in the nature of the products. Thus C-2 epimers will yield D- or L-glyceraldehyde, C-3epimers will give hexoses or hexose methyl ethers, and C-4 epimers will result in the formation of erythritol or threitol.

Determination of the carbonyl compounds amongst the products of the Smith degradation presents a problem. Thus, conventional Smith-degradation may yield glycolaldehyde and glyceraldehyde each of which dimerizes[7], whereas the modified method can give rise to 3-*O*-methylglyceraldehyde which also dimerizes. Anomeric methyl pyranosides and/or furanosides may be formed from partially methylated hexoses and characterized if required as the acetates, trifluoroacetates, *O*-trimethylsilyl derivatives, or *p*-iodobenzoates. A simpler procedure involves reduction with sodium borohydride of the products obtained after hydrolysis of the methylated polyalcohol followed by determination of the methylated alditols. Thus, methyl glycolaldehyde yields 2-methoxyethanol, the formation of which indicates the presence of H*p*1- and -6H*p*1- bond types. Likewise, 3-*O*-methylglyceraldehyde, formed from -2H*p*1- bond types, yields 1-*O*-methylglycerol, the D form of which could, for example, derive from D-mannose and the L form from D-glucose.

Partially methylated hexoses would, of course, be converted into partially methylated hexitols.

The applicability of the modified Smith-degradation method was investigated with two clinical dextrans (Plasmodex and Polfa). The molecular weight of each

dextran was determined according to the methods of Isbell[8] and Unrau[9], and the values were used in calculating the periodate consumption (determined photometrically[10] and iodometrically[11]) and the release of formic acid (determined iodometrically[12]). The results are shown in Table I.

TABLE I

PERIODATE OXIDATION OF CLINICAL DEXTRANS

Dextran	*Mol. wt.*	*Periodate consumption (mol./Glc residue)*	*Formic acid released (mol./Glc residue)*
Plasmodex	39,500[a]; 41,200[b]	1.90[c]; 1.90[d]	0.92
Polfa	43,600[a]; 46,000[b]	1.91[c]; 1.92[d]	0.94

[a]Determined by Isbell's method[8]. [b]Determined by Unrau's method[9]. [c]Determined spectrometrically[10]. [d]Determined iodometrically[12].

Each glucan was methylated three times by the Haworth method and then once by the Kuhn procedure[13]. The extent of methylation was followed on the basis of i.r. absorption for hydroxyl groups in the range 3400–3600 cm^{-1}. The final methoxyl contents were 45.35 and 45.29%, respectively (theoretical: 45.6%). After hydrolysis with formic acid–sulphuric acid[14], the products were separated by t.l.c. and quantitatively determined by using the aniline hydrogen phthalate[15] and anthrone[16] reagents. For Plasmodex, the molar proportions of 2,3-di-, 2,4-di-, 2,3,4-tri-, and 2,3,4,6-tetra-*O*-methyl derivatives of D-glucose were 5.9:1.9:84.3:7.9, whereas, for Polfa, 2,4-di-*O*-methyl-D-glucose was not detected, and the proportions of 2,3-di-, 2,3,4-tri-, and 2,3,4,6-tetra-*O*-methyl derivatives of D-glucose were 6.1:87.7:6.2. Thus, Plasmodex has the more-branched molecule and has (1→4)- and (1→3)-branch points, whereas only (1→4)-branch points are found in the Polfa dextran.

Each glucan was subjected in sequence to periodate oxidation and borohydride reduction. The resulting polyalcohol was methylated first by the Haworth procedure and then by the Kuhn method. The methylated polyalcohols were then hydrolysed with formic acid and sulphuric acid, and the products were reduced with sodium borohydride. Trimethylsilylation[17] of the reduced products, followed by g.l.c., revealed the formation of 2,4-di-*O*-methylglucitol, 1-*O*-methylerythritol, and 1-*O*-methylglycerol from Plasmodex. It was not possible to separate 1,3-di-*O*-methylglycerol and 2-methoxyethanol from the reaction mixture formed on trimethylsilylation. 1-*O*-Methylerythritol and 1-*O*-methylglycerol were formed from the Polfa dextran; the glucitol methyl ether could not be detected.

If acetates rather than trimethylsilyl ethers were used for g.l.c.[18], it then proved possible to detect and identify the degradation products of lower molecular weight (Table II).

According to the results obtained by the three different methods described above, each glucan has a linear structure to an extent of 90–93%. However, an essential difference is a small percentage (2–3%) of α-(1→3)-branch points which

occur in the Plasmodex dextran. This can be established both from methylation analysis and the results of the modified Smith-degradation.

TABLE II

G.L.C. DATA[a] FOR THE REDUCED PRODUCTS OF THE MODIFIED SMITH-DEGRADATION

Reduced products	*Trimethylsilyl ether*				*Acetate*			
	Retention time (min)		*Mol. proportion (%)*		*Retention time (min)*		*Mol. proportion (%)*	
	A	B	*Plasmodex*	*Polfa*	C	D	*Plasmodex*	*Polfa*
2-Methoxyethanol	—	—	—	—	3.6	3.9	47.6	50.0
1,3-Di-*O*-methylglycerol	—	—	—	—	4.8	5.8	3.8	3.4
1-*O*-Methylglycerol	0.7	1.2	46 6	44.2	5.2	6.2	42.2	43.5
1-*O*-Methylerythritol	3.3	4.9	3.0	3.4	8.9	11.9	2.6	3.1
2,4-Di-*O*-methylglucitol	21.6	19.9	2.4	—	—	—	—	—

[a]G.l c. conditions: *A* 3% SE-30 at 180°; *B* 3% SE-52 at 200°; *C* 20% Apiezon M and 20% butanediol succinate[18] at 80°; *D* 4% XE-60 at 60°. The phases *A–D* were supported on Chromosorb W.

EXPERIMENTAL

U.v. and visible-range spectra were obtained with a Beckman DB spectrophotometer, and i.r. spectra with a UKC-14 instrument. Methylated hexoses were separated by t.l.c. on Kieselgel G (Merck), using butanone saturated with water. G.l.c. was effected with a CHROM-3 IKZ instrument with flame-ionization detection and automatic integration of peak areas. Nitrogen (120 ml/min) was the carrier gas, and a column (2 m × 5 mm, i.d.) of stainless steel (form W) was used. The packings are noted in Table II.

Methylation of dextrans. — A solution of Plasmodex (2 g) in water (20 ml) was basified with 1.70 ml of 30% aqueous sodium hydroxide. 30% Aqueous sodium hydroxide (6.7 ml) and freshly distilled methyl sulphate (2.7 ml) were then added simultaneously at room temperature with stirring during 1 h. After a further 1 h, the reaction mixture was dialysed against water for 24 h and then evaporated to dryness at 40° (bath) *in vacuo*.

A solution of the residue in water (20 ml) was subjected to a second methylation as described above, except that the reaction mixture was stirred at 45° for 1 h after the addition of the methylation reagents. In the third methylation, the corresponding temperature was 75°. The final product (1.75 g) was a glass soluble in chloroform and *N*,*N*-dimethylformamide (Found: OMe, 30.2%).

A solution of the partially methylated dextran (1.75 g) in *N*,*N*-dimethylformamide (20 ml) was treated overnight with methyl iodide (4.1 ml) and active silver oxide (4.1 g). The mixture was then diluted with chloroform (100 ml) and filtered, and the insoluble material was washed with chloroform (3 × 10 ml). The combined,

filtrate was washed with 1% aqueous potassium cyanide (2 × 30 ml) and water (4 × 60 ml), dried (Na_2SO_4), and evaporated. *N,N*-Dimethylformamide was removed from the residue by evaporation of ethanol (3 × 10 ml) therefrom. A portion (1.36 g) of the product (1.5 g; OMe, 41.8%) was again methylated with *N,N*-dimethylformamide (30 ml), methyl iodide (3.14 ml), and silver oxide (3.14 g) as described above. Traces of *N,N*-dimethylformamide were removed from a portion (1 g) of the syrupy product (1.28 g; OMe, 44.3%) by elution from a column (diameter, 2 cm) of neutral alumina (30 g), using chloroform–benzene (1:1, 500 ml). Evaporation of the eluate gave the product as a white, amorphous solid (0.85 g; OMe, 45.35%) which had no i.r. absorption for hydroxyl at ~3630 cm^{-1}.

The Polfa dextran was methylated in a similar manner. After five methylations, the product had OMe, 42.85% and, after a further methylation and removal of *N,N*-dimethylformamide as described above, this value was raised to 45.29%.

Hydrolysis of the methylated dextrans. — A solution of methylated Plasmodex (0.6 g) in 90% formic acid (25 ml) was maintained at ~95° (boiling water-bath) for 1 h and then evaporated *in vacuo*. The syrupy residue was dissolved in 0.25M sulphuric acid (25 ml), and the solution was kept at 100° for 14 h. The cooled solution was neutralized ($BaCO_3$) and then centrifuged at 3000 r.p.m. for 15 min after dilution with water (100 ml). The precipitate was suspended in 96% ethanol (125 ml) and centrifuged again. The two supernatants were combined, filtered, and evaporated to give a syrup (506 mg), a solution of a portion (0.4 g) of which in 96% ethanol (10 ml) was used for chromatographic examination. Hydrolysis of methylated Polfa dextran (600 mg) under similar circumstances gave 520 mg of syrup.

The hydrolysates were analysed as follows: (*a*) Portions (100, 200 μg) were separated on sheets (20 × 20 cm) of Kieselgel G with the solvent front running to 15 cm. The dried sheets were then treated[15] with aniline hydrogen phthalate in ethanol at 105°, and the intensity of the colours generated was measured at 415 nm.

(*b*) After similar chromatography of quantities (30, 50, and 100 μg) of hydrolysate using appropriate markers, the adsorbent in the zones containing the methyl ethers was removed, suspended in water (1 ml), cooled, and mixed with anthrone reagent[16] (1 ml). After heating at ~95° for 15 min, the solutions were cooled and centrifuged, and the colour which had developed in the supernatant was measured at 625 nm.

Modified Smith-degradation. — A solution of the dextran (0.5 g) in water (50 ml) was treated with sodium metaperiodate (2.66 g), and the volume was made up to 100 ml. The solution was stored at 5° in the dark. After 76 h, the consumption of periodate was 1.90 mol. for Plasmodex and 1.92 mol. for the Polfa dextran, and the formic acid released was 0.92 and 0.94 mol., respectively. To each reaction mixture, ethylene glycol (0.5 ml) was added and, after 2 h, the mixture was dialysed against water for 48 h. The dialysed solutions were made 80% with respect to methanol, and the precipitated gum was collected and washed well with 80% aqueous methanol. The resulting polyaldehyde (0.32–0.34 g) was dissolved in water (25 ml) and reduced by the addition of a solution of sodium borohydride (0.5 g) in water (5 ml). After 24 h,

the mixture was neutralized with glacial acetic acid and evaporated *in vacuo* at 40°. Methanol (3 × 20 ml) was distilled from the residue to give the polyalcohol (~300 mg).

A solution of the polyalcohol (300 mg) in 30% aqueous sodium hydroxide (3 ml) was stirred, and during 3 h 30% sodium hydroxide (30 ml) and methyl sulphate (10 ml) were added simultaneously. The mixture was then kept at 90° for 1 h and after cooling, extracted with chloroform (6 × 30 ml). The combined extracts were washed with water until neutral, then dried, and evaporated. A solution of the residue in *N*,*N*-dimethylformamide (8 ml) was methylated with methyl iodide (2 ml) and silver oxide (2 g) for 24 h. More methyl iodide (2 ml) and silver oxide (2 g) were added, and stirring was continued for a further 24 h. The mixture was then diluted with chloroform (100 ml), filtered, and washed with 1% aqueous potassium cyanide (3 × 10 ml) and water (5 × 20 ml), dried, and evaporated. The resulting methylated polyalcohols were syrups: 215 mg (OMe, 38.47%) from Plasmodex and 198 mg (OMe, 38.29%) from Polfa.

Portions (150 mg) of the methylated alcohols were hydrolysed with 82% formic acid (18 ml) at 100° for 1 h. The hydrolysates were evaporated, and the residues were dissolved in 0.5M sulphuric acid (10 ml) and kept at 100° for 14 h. The reaction mixtures were diluted with water (20 ml), de-ionized with Amberlite IR-120(H^+) and IR-45B(HO^-) resins (10 ml each), and concentrated to 5 ml. Sodium borohydride (50 mg) was added, and after 2 h the solutions were neutralized with glacial acetic acid, de-ionized, and evaporated with methanol (3 × 5 ml). The syrupy products (114 mg from Plasmodex and 120 mg from Polfa) were dried over phosphorus pentaoxide and potassium hydroxide.

For g.l.c., portions (10 mg) of the hydrolysates were dissolved in pyridine (1 ml), and hexamethyldisilazane (0.2 ml) and chlorotrimethylsilane (0.1 ml) were added. After stirring for 1 min and storage for 5 min, the mixtures were centrifuged (3,000 r.p.m.), and portions (0.5–5 μl) of the upper layers were injected on to columns *A* and *B* at 240°.

In parallel experiments, portions (10 mg) of the hydrolysates were dissolved in pyridine (0.2 ml) and acetic anhydride (0.2 ml). After storage at 100° for 3 h and then cooling, portions (1–5 μl) were analysed on columns *C* and *D*.

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