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LINKAGE ANALYSIS OF (1→6)-LINKED OLIGOSACCHARIDES BY ALKALINE DEGRADATION

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ABSTRACT

2-*C*-Methylglyceric acid was formed by sequential degradation of the chain in isomaltose oligosaccharides in aqueous barium hydroxide. The products of the stopping reaction, namely 6-*O*-substituted 3-deoxy-D-hexonic acids, were also obtained in considerable yield. The separation and determination of these products is of importance for the analysis of the linkage sequence in oligosaccharides.

INTRODUCTION

The formation of saccharinic acids on degrading oligosaccharides in alkaline solution is known to reflect the nature of the inter-unit linkages. Thus, 3-*O*-substituted hexoses give primarily 3-deoxyhexonic acids on anaerobic, alkaline degradation[1–3], whereas 4-*O*-substituted hexoses give mainly 3-deoxy-2-*C*-(hydroxymethyl)pentonic acids[1,4,5]. 1-*O*-Substituted hexuloses give 2-*C*-methylpentonic acid[1,6], whereas 2-*O*-substituted hexoses are relatively stable in alkaline solutions[1]. No such specific product has been reported for the degradation of 6-*O*-substituted hexoses[7,15]. Such a product is now described.

DISCUSSION

Melibiose is known[7,8] to be degraded in aqueous, alkaline solution to give lactic acid (**1**), 3-deoxy-6-*O*-α-D-galactopyranosyl-D-*ribo*-hexonic acid (**2**), and 3-deoxy-6-*O*-α-D-galactopyranosyl-D-*arabino*-hexonic acid (**3**). Products **2** and **3** are formed by a "stopping" reaction in which a hydroxyl ion, in preference to a galactose residue, is lost in the β-elimination stage of the degradation, with the resultant formation of an alkali-stable residue (Fig. 1). The glycosidic linkage thus remains intact. Such a stopping reaction is thought to be responsible for the formation of alkali-stable end-groups in the degradation of dextran[9]. Cleavage of the glycosidic linkage in melibiose occurs with formation of lactic acid[7]. The reducing D-glucose moiety of the disaccharide is cleaved between C-3 and C-4. Subsequent rearrangement of the triose products results in the elimination of galactose and the production of lactic acid (Fig. 1).

In a recent examination[8] of the product mixture obtained on degrading meli-

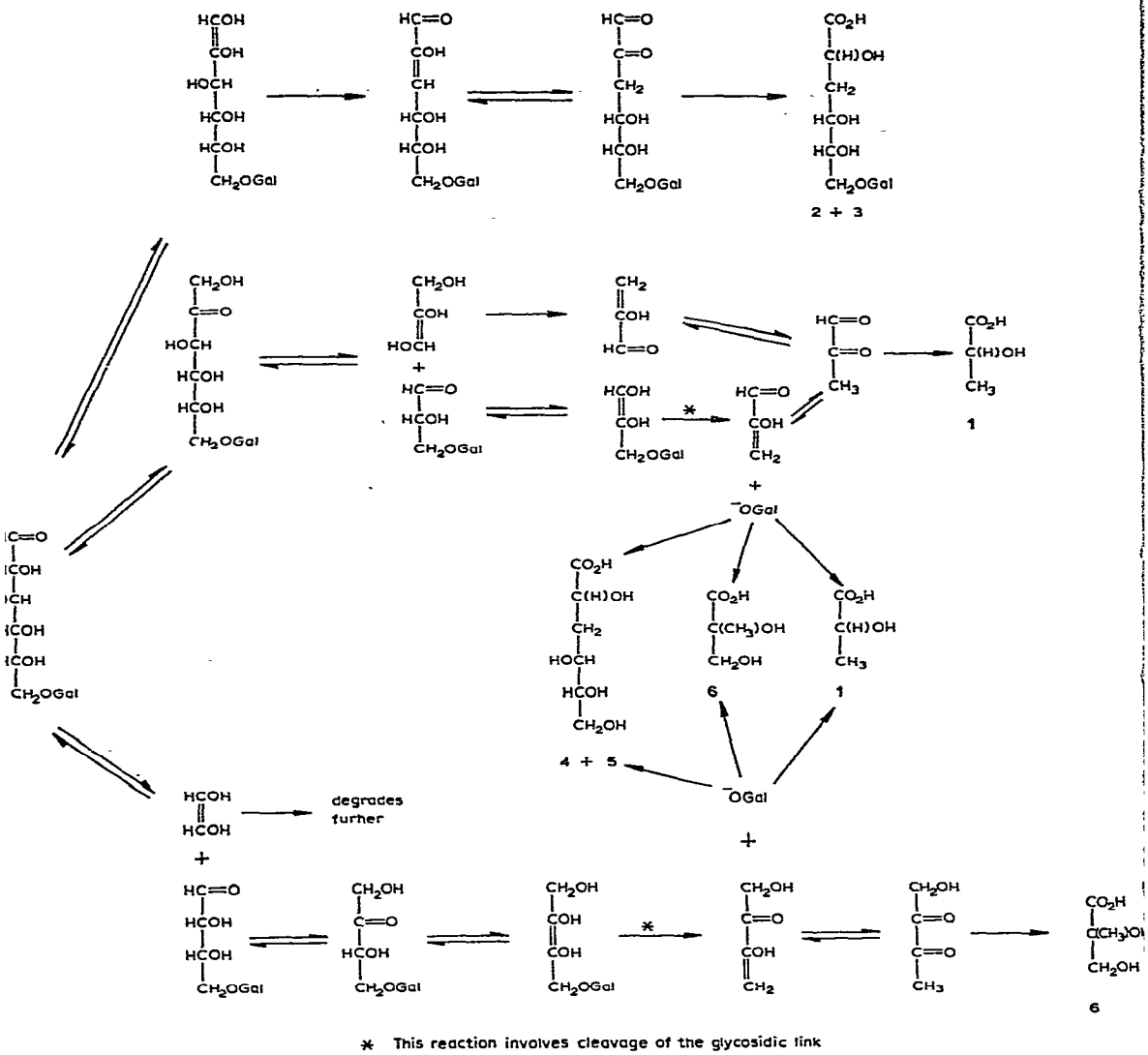

Fig. 1. Degradation of melibiose.

biose anaerobically in aqueous barium hydroxide, considerable amounts of 3-deoxy-D-*xylo*-hexonic acid (**4**), 3-deoxy-D-*lyxo*-hexonic acid (**5**), and 2-*C*-methylglyceric acid (**6**) were detected in addition to **2** and **3**[8]. Degradation of the released galactose accounted for the presence of **4** and **5** (Fig. 1), but the origin of **6** was less obvious. It was suggested[8] that cleavage of the C-3–C-4 bond in the D-glucose moiety of the disaccharide would yield a 4-*O*-substituted tetrose which could then be degraded with production of **6** and release of D-galactose (Fig. 1). Such a reaction mechanism does not preclude the formation of **6** from the subsequent degradation of the released galactose. Indeed the latter mode of formation must be considered as a possible

source of **6** in view of the observation that glucose is known to readily form both tetrose and triose fragments under alternative reaction conditions[10]. Nevertheless, if **6** is formed at least partially from the D-glucose moiety of melibiose, it follows that 6-*O*-substituted hexose oligosaccharides should be degraded sequentially in alkaline solutions with formation of both **6** and **1**.

To test this hypothesis, isomaltose, isomaltotriose, and isomaltotetraose were each degraded in aqueous barium hydroxide. The degradation products were fractionated by anion-exchange chromatography and the amount of **6** was determined. The products of the stopping reaction, the 6-*O*-substituted 3-deoxy-D-hexonic acids, were also detected, as were 3-deoxy-D-*ribo*-hexonic acid (**7**) and 3-deoxy-D-*arabino*-hexonic acid (**8**). Product **1** was not determined quantitatively. The degradation products of D-glucose were also examined, as D-glucose would obviously be released and subject to subsequent degradation in each of the reaction mixtures. Compounds **7** and **8** were found in the degradation products of each of the four substrates, together with large proportions of **6**.

D-Glucose-*6-t* was also degraded. The reaction products were fractionated by anion-exchange chromatography, with scanning for radioactivity. Products were detected corresponding in elution position with **1**, **6**, **7**, and **8**. Those fractions corresponding to **6** were re-fractionated to remove the large amounts of **1** also present. Compound **6** so obtained, which was radioactively labelled, was oxidised with periodic

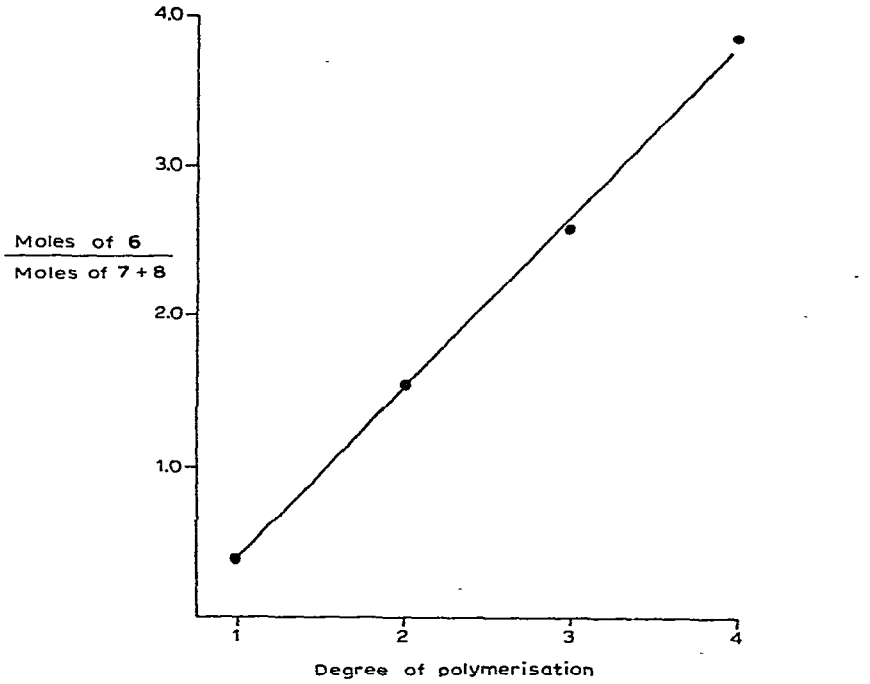

Fig. 2. Increase in production of 2-*C*-methylglyceric acid with increase in degree of polymerisation.

acid to give pyruvic acid which on further anion-exchange chromatography was also shown to be radioactive. The proposed[8] mechanism of formation of **6** predicts that the tritium-labelled C-6 unit of D-glucose-*6-t* should appear at C-4 of **6** and C-3 of pyruvic acid. This was indeed found to be the case, with the activities of the D-glucose-*6-t*, **6**, and pyruvic acid being closely similar (±5%).

Degradation of both unlabelled and labelled D-glucose gave **6**, **7**, and **8**. Each of these compounds was also found in the degradation products of isomaltose, isomaltotriose, and isomaltotetraose, but **7** and **8** could only have arisen from the degradation of D-glucose released in the course of each reaction. Assuming that the D-glucose released in each reaction is degraded to give similar molar yields of **6**, **7**, and **8**, the amount of **7** and **8** formed defines the amount of **6** produced from the non-reducing D-glucose residue of each substrate. The relative proportions of these three products (for each of the four degradations) are shown in Fig. 2. It will be seen that the relative yield of **6** from isomaltose is higher than that from D-glucose alone, suggesting that **6** arises from both units of the disaccharide. Furthermore, the relative yield of **6** increases linearly in the degradation of isomaltotriose and again in the degradation of isomaltotetraose. By assuming that isomaltose released in the degradation of both isomaltotriose and isomaltotetraose is degraded to give relative yields of products similar to those produced by degrading isomaltose alone, and by making similar assumptions about the degradation of free and released isomaltotriose, the data expressed in Fig. 2 are consistent with formation of **6** by degradation of each successive unit in the oligosaccharide chain.

6-*O*-Substituted 3-deoxyhexonic acids were also obtained. Isomaltose gave the 6-*O*-D-glucosyl acid (**9**) whilst isomaltotriose gave the 6-*O*-isomaltosyl acid (**10**) in addition to **9**. Isomaltotetraose gave both **9** and **10**, together with the 6-*O*-isomaltotriosyl acid (**11**).

It has been suggested[11] that, in the sequential degradation of cellulose in alkali, several hundred D-glucose units may be removed from the polysaccharide chain before alkali-stable end-groups are produced by a stopping mechanism similar to that reported herein. The yields of stopping-reaction products obtained here are thus somewhat higher than might have been expected.

Important structural information can be deduced from the presence of such 6-*O*-substituted 3-deoxyhexonic acids amongst the products of alkaline degradation because the number of monosaccharide units present in their glycosyl residues indicates the position of (1→6)-linkages in the oligosaccharide undergoing degradation. Thus, Fig. 3 shows the anticipated degradation products from an oligosaccharide containing both (1→3)- and (1→6)-linkages. In the 6-*O*-substituted 3-deoxyhexonic acids produced in the present degradations, the numbers of D-glucose residues in the glycosyl residues were readily determined by anion-exchange chromatography under conditions shown to separate a mixture of lactobionic, 6-*O*-isomaltosyl-D-gluconic, and 6-*O*-isomaltotriosyl-D-gluconic acid.

Thus, dextran oligosaccharides are degraded in alkali to produce **6** by a peeling mechanism. 6-*O*-Substituted 3-deoxy-D-hexonic acids are produced concurrently

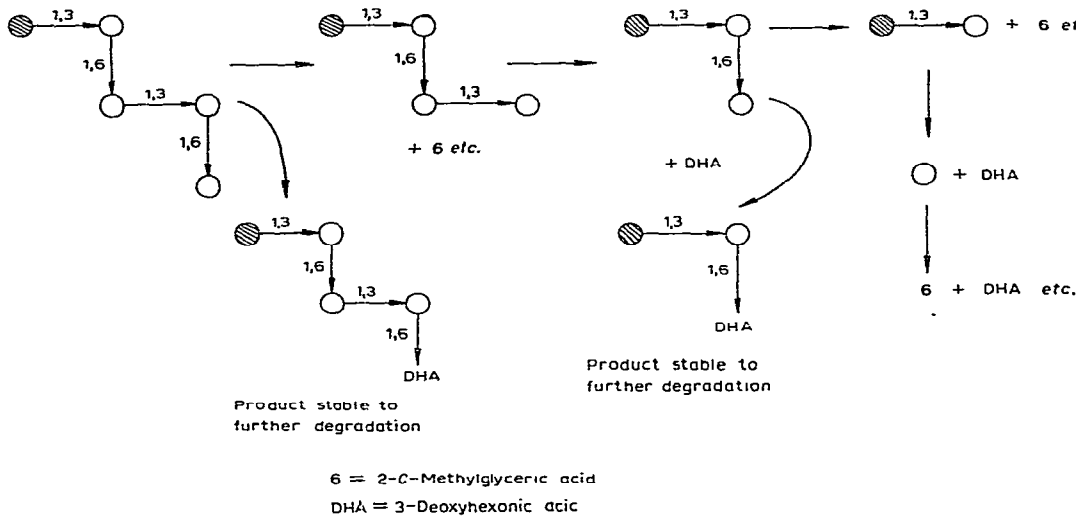

Fig. 3. Postulated degradation of a pentasaccharide containing both (1→3)- and (1→6)-linkages, with the formation of alkali-stable 6-*O*-glycosyl derivatives of 3-deoxyhexonic acids indicating the linkage sequence.

by a stopping reaction. Sensitive spectrophotometric methods[12,13] and radiochemical-labelling techniques[14] already exist which will enable such 6-*O*-substituted 3-deoxyhexonic acids to be distinguished from other *O*-substituted aldonic or *O*-substituted deoxyaldonic acids which may be formed on degrading oligosaccharides containing more than one type of linkage. The application of the method of linkage analysis described here to the analysis of oligosaccharides is being studied.

EXPERIMENTAL

Preparation of model substrates. — Isomaltose, isomaltotriose, and isomaltotetraose were prepared by hydrolysis of dextran (3.0 g; *Leuconostoc mesenteroides* B512) with sulphuric acid (165mM, 100 ml; 6 h at 100°). After neutralisation, the reaction product was fractionated on a large column of cation-exchange resin (Dowex-50W X2; 200–400 mesh; lithium form)[16]. The column eluate was monitored continuously for carbohydrate[16]. Fractions containing isomaltose, isomaltotriose, and isomaltotetraose, respectively, were freeze-dried, and samples of each of these materials were examined by paper chromatography (Whatman No. 1; descending eluant, 1-butanol–pyridine–water, 6:4:3), using silver nitrate[17] for detection.

Degradation of model substrates. — Samples of D-glucose (2.0 mg), isomaltose (2.0 mg), isomaltotriose (3.8 mg), and isomaltotetraose (4.1 mg), together with solid barium hydroxide, were added to water (carbohydrate concentration, 2.0 mg/ml; barium hydroxide 0.25M). Dry, oxygen-free nitrogen was then bubbled through the mixture for 10 min to remove residual oxygen. The reaction vessels, still containing nitrogen, were then sealed and kept at 50° for 3 h. The resulting solutions were freeze-

dried, and water (2.0 ml) was added to the residues. Aliquots (1.5 ml) were then fractionated on resin column *A* (Dowex-AG1 X8; 200–400 mesh; acetate form; 140.0 × 0.6 cm; bed volume, 37 ml) by elution with deaerated 0.5M acetic acid[18] (0.80 ml/min). The remaining aliquots (0.5 ml) were fractionated on resin column *B* (Dowex-AG1 X8; 200–400 mesh; acetate form; 66.0 × 0.5 cm; bed volume, 15 ml) by elution with an ammonium acetate gradient. Samples were washed on to column *B* with water (30 ml; 0.6 ml/min), and ammonium acetate was added to the column eluant to produce a concentration gradient up to 0.2M (100 ml; 0.60 ml/min), which was then maintained (30 ml, 0.60 ml/min). The eluates from columns *A* and *B* were continuously monitored by using the automated cysteine–sulphuric acid[19] and fluorimetric formaldehyde[20] methods. The results shown in Fig. 2 exemplify an elution from column *A*.

Degradation of D-*glucose*-6-t. — An aliquot (0.1 ml) of an aqueous solution of D-glucose-*6-t* (500 mCi/mmole, 50 mCi/ml; Radiochemical Centre, Amersham) was added to D-glucose (2.0 mg) and the mixture degraded as above. The total reaction product, after freeze-drying, was fractionated on an anion exchanger by elution with acetic acid as described above. The column eluate was scanned for radioactivity, and those fractions corresponding in elution position with **6** were combined and freeze-dried. Lactic acid was removed from the sample by anion-exchange chromatography in ammonium acetate as described above. The resulting **6** was freeze-dried and the solid taken up in water (2.0 ml). This solution was added to periodic acid (4.0 ml; 25mM in 62.5mM H_2SO_4) and the mixture was kept at room temperature for 20 min. Excess barium carbonate was added and the resulting precipitate removed by centrifugation. The supernatant solution was then fractionated on a resin column (Dowex-AG1 X8; 200–400 mesh; formate form; 22.0 × 0.5 cm; bed volume, 5 ml) by elution with formic acid of increasing strength (linearly increasing from 0 → 6M; 100 ml; 0.60 ml/min). The eluate was monitored for radioactivity. This chromatographic system was calibrated with pyruvic acid, and the elution positions of lactic acid and glyoxylic acid were known[21].

Liquid scintillation counting. — Samples (1.0 ml) were emulsified on addition to a Triton X-100/toluene mixture (10.0 ml; 2:1) containing 2-(4′-*tert*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole (butyl-PBD) (15 g/l; Koch–Light Laboratories Ltd.) and counted at 10° on an ABAC SL40 instrument.

6-*O*-Isomaltosyl-D-gluconic acid and 6-*O*-isomaltotriosyl-D-gluconic acid were prepared by alkaline hypoiodite oxidation of isomaltotriose and isomaltotetraose, respectively[22], and were used for chromatographic comparisons.

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