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STUDIES ON DEXTRANS AND DEXTRANASES

PART XI[1]. THE STRUCTURE OF A DEXTRAN ELABORATED BY *Leuconostoc mesenteroides* NRRL B-1299

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ABSTRACT

In dextran *S* elaborated by *Leuconostoc mesenteroides* NRRL B-1299, the polymeric chain consists, principally, of segments of isomaltose homologues which are mutually linked through positions 1 and 2 of their terminal D-glucose residues. The average repeating-unit, containing a total of fifteen D-glucose residues, possesses five branches which occur at each position 6 of such segments and at positions 2. Branches consist mainly of α-D-glucopyranosyl groups and some appear to be terminated by α-nigerosyl groups.

INTRODUCTION

Analysis of the complex mixtures of oligosaccharides produced by the action of dextranases on native dextrans possessing secondary linkages has afforded an insight into both the patterns of the action of the enzymes[2] and the structures of these dextrans[3,4]. We now report the results of studies on the action of the dextranase of *Penicillium lilacinum* (CMI 79197; NRRL 896) (dextranase A of Part II[5]) on the native, water-soluble dextran (dextran *S*) of *Leuconostoc mesenteroides* NRRL B-1299 and on the acid-degraded dextran *S* (dextran *S-AD*). Of the reports concerning the structure of the dextrans of *L. mesenteroides* NRRL B-1299, the following are pertinent to the present investigation. Jeanes and associates[6] concluded, on the grounds of statistical analysis of products of low molecular weight obtained by partial hydrolysis with acid, that most of the external branches in dextran *S* consist of single α-D-glucopyranosyl residues. Immunochemical studies by Kabat and co-workers[7–11] have indicated that terminal glucosidic linkages on non-reducing chain ends in dextran *S* and dextran *L* (the less-soluble fraction produced by *L. mesenteroides* NRRL B-1299) are most frequently of the α-(1→2) type and that others are of the α-(1→3) and α-(1→6) types. We have recently reported[1] that the approximate number of terminal, non-reducing D-glucose residues and those linked through positions 1 and 6, 1 and 3, as well as 1, 2, and 6 in the average repeating-unit of dextran *S* are 5, 4, 1, and 5, respectively. The corresponding figures for dextran *L* are 5, 4, 3, and 5.

RESULTS AND DISCUSSION

Dextran *S* was only slightly degraded by dextranase A. This result was expected in view[5] of the high content (41%) of α-D-glucopyranosyl residues linked through secondary linkages; the same result was obtained with the dextranase of *P.funiculosum* (C.M.I. 79195; NRRL 1132) (dextranase B of Part II[5]). However, partial hydrolysis with acid produced a polymeric material (dextran *S-AD*) which retained a proportion of secondary linkages, and yet was readily degraded by dextranase A. Conditions for obtaining such a non-dialysable material in reasonable yield were obtained from a series of trial experiments. The results of periodate oxidation suggested that all (1→3)-linkages in dextran *S* were hydrolysed during its acidic degradation, and that 9% of α-D-glucopyranosyl residues in dextran *S-AD* were linked through secondary linkages of the (1→2) type (the method[12] does not distinguish between residues linked through positions 1 and 2, and positions 1, 2, and 6), the remainder being terminal, non-reducing residues and residues linked through primary linkages only.

Paper chromatography of a dextranase–dextran *S-AD* digest revealed, in addition to products expected[2] from sections of dextran molecules possessing only primary linkages (*i.e.*, D-glucose, isomaltose, and isomaltotriose), several higher saccharides, three of which were isolated by methods similar to those described earlier[3,4]. The assignment of their molecular size was made on the basis of reducing power (Table I). The molecular size of the heptasaccharide *A* was deduced from its rate of chromatographic migration. Tetrasaccharide *A* and pentasaccharide *A* were selected for further studies.

TABLE I

YIELDS AND PROPERTIES OF PRODUCTS FROM DEXTRAN *S-AD* PRODUCED BY DEXTRANASE A

Product	*Yield (g from 50 g of dextran* S-AD)	*D.p.*[a]	$[\alpha]_D^{20}$ [b] *(degrees)*	R_G	M_G(B)	M_S(Mo) [c]
D-Glucose	0.60			1.00	1.00	1.00
Isomaltose	10.20	1.9	+120	0.74	0.70	0.70
Isomaltotriose[d]	7.10	2.9		0.56	0.60	0.58
Tetrasaccharide *A*	1.10	4.0	+157	0.42	0.50	0.46
Pentasaccharide *A*	1.60	5.0	+169	0.26	0.45	0.41
Hexasaccharide *A*	0.60	5.9	+171	0.16	0.41	0.38
Heptasaccharide *A*	Not measured	—	—	0.11	—	—

[a]Determined by the method of Timell[23]. [b]In water (*c* 0.6–0.7). [c]After reduction with $NaBH_4$. [d]This fraction contained a trace component, possibly a trisaccharide, with R_G 0.60.

Electrophoresis of a saccharide and its reduction product in borate and molybdate solutions, respectively, can be used for determining the position of the glycosidic linkage to the reducing unit of the original saccharide[13]. Accordingly, the

rates of electrophoretic migration of the tetra- and penta-saccharides *A* in borate solution and of their reduction products in molybdate solution (Table I) show that the reducing D-glucose residues in the oligosaccharides are linked only through position 6.

The results of oxidation with periodate of the tetra- and penta-saccharides *A* (Table II) are those expected from oligosaccharides possessing one (1→2)-linkage to units other than the reducing D-glucose residue, the remainder being (1→6)-linkages; methylation analysis of dextran *S* and the oligosaccharides (see below) rule out the possibility of a (1→4)-linkage.

TABLE II

PERIODATE OXIDATION OF OLIGOSACCHARIDES

Oligosaccharide	*Periodate reduced (mol.)*		*Formic acid produced (mol.)*	
	Found	*Calc.*[a]	*Found*	*Calc.*[a]
Tetrasaccharide *A*	9.0	9.0	5.9	6.0
Pentasaccharide *A*	10.8	11.0	6.9	7.0

[a]Calculated for oligosaccharides possessing one (1→2)-linkage, but not to the reducing D-glucose residue, the remainder being (1→6)-linkages.

G.l.c. of the methanolysates of the methylated tetra- and penta-saccharides *A* revealed components having retention volumes identical with those of the methyl α- and β-pyranosides of 2,3,4,6-tetra-, 2,3,4-tri-, and 3,4,6-tri-*O*-methyl-D-glucoses. On the evidence presented so far, the possible structures for tetrasaccharide *A* are **1** and **2**, and those for pentasaccharide *A* are **3**, **4**, and **5** (Fig. 1). The structures were

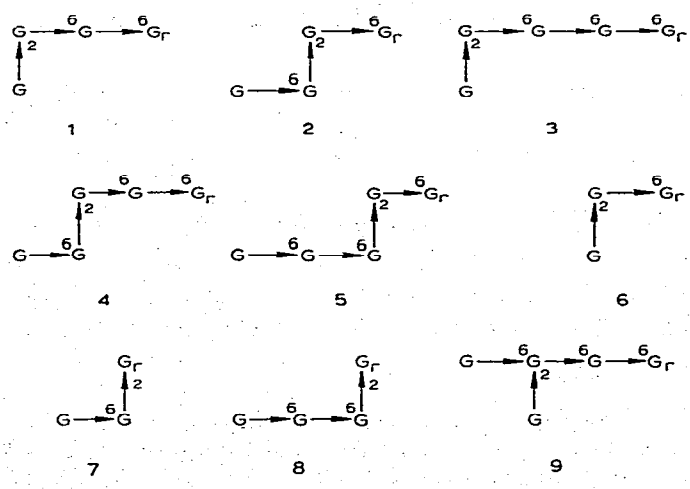

Fig. 1. Structure of oligosaccharides. G, α-D-glucopyranosyl unit; G_r, reducing D-glucose unit.

further elucidated by fragmentation by acetolysis and acid hydrolysis, and examination of products by paper electrophoresis and chromatography (Table III).

TABLE III

PRODUCTS OF ACETOLYSIS OF TETRASACCHARIDE *A* AND PENTASACCHARIDE *A*

Fraction	R_G	$M_G(B)$	$M_S(Mo)$ [a]	*Identity*
1	1.00	1.00	1.00	D-Glucose
2	0.83	0.30	0.70	Kojibiose
3	0.74	0.70	0.70	Isomaltose
4	0.56	(i) 0.60	0.58	Isomaltotriose and **6**
		(ii) 0.27		**7**
5[b]	0.42	0.50	0.46	Isomaltotetraose, **1**, and **2**

[a]After reduction with $NaBH_4$. [b]Only from pentasaccharide *A*.

Tetrasaccharide A. — The characteristic fragments of the tetrasaccharides **1** and **2** are isomaltotriose and *O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-D-glucose (**7**), respectively. The trisaccharide fraction obtained by acetolysis, followed by deacetylation, contained two components 4(i) and 4(ii), which had $M_G(B)$ 0.60 and 0.27, respectively, expected of trisaccharides in which the reducing D-glucose residue is linked through position 6 [isomaltotriose and *O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→6)-D-glucose (**6**)] and position 2 (**7**), respectively*. Since the component with $M_G(B)$ 0.60 stained far more intensely than that with $M_G(B)$ 0.27, and as the trisaccharide **6** can arise from both tetrasaccharides **1** and **2**, it is likely that the faster-migrating component contained isomaltotriose and trisaccharide **6**. It is thus concluded that tetrasaccharide *A* is a mixture of *O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→6)-D-glucose (**1**) and *O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→6)-D-glucose (**2**).

Pentasaccharide A. — The tetrasaccharide fraction (5), obtained from pentasaccharide *A* by acetolysis followed by deacetylation, migrated during eletrophoresis in borate solution as a single component with $M_G(B)$ 0.50, expected of tetrasaccharides in which the reducing D-glucose residue is linked through position 6. As a component with an $M_G(B)$ value <0.27, expected of *O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-D-glucose (**8**), could not be detected, it is likely that the pentasaccharide *A* contained only insignificant quantities, if any at all, of pentasaccharide **5**. This conclusion is in agreement with the action

*Since this work was completed[14], Sakakibara *et al.*[15] have obtained *O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→6)-D-glucose (**6**) and *O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-D-glucose (**7**) by acetolysis of the dextran of *L. mesenteroides* NRRL B-1397, and have reported rates of electrophoretic migration that are virtually identical with those recorded in this paper.

pattern[2] of dextranase *A*. If liberated from dextran *S-AD*, pentasaccharide **5** would be hydrolysed by dextranase A to give isomaltose and trisaccharide **6**.

The trisaccharide fraction obtained by the above fragmentation could be resolved to give the same components as were obtained from tetrasaccharide *A*, *i.e.*, 4(i) and 4(ii). As structure **5** has been eliminated, the component with $M_G(B)$ 0.27 (**7**) undoubtedly arises from pentasaccharide **4**.

Acid hydrolysis of pentasaccharide *A* gave, *inter alia*, a tetrasaccharide fraction which was isolated by paper chromatography. Treatment of this material with dextranase A resulted in rapid and extensive degradation, giving isomaltose as the main product. The acid hydrolysate thus contained appreciable quantities of isomaltotetraose which, as indicated by the other evidence, could arise only from pentasaccharide **3**. Pentasaccharide *A* was thus a mixture of *O*-α-D-glucopyranosyl-

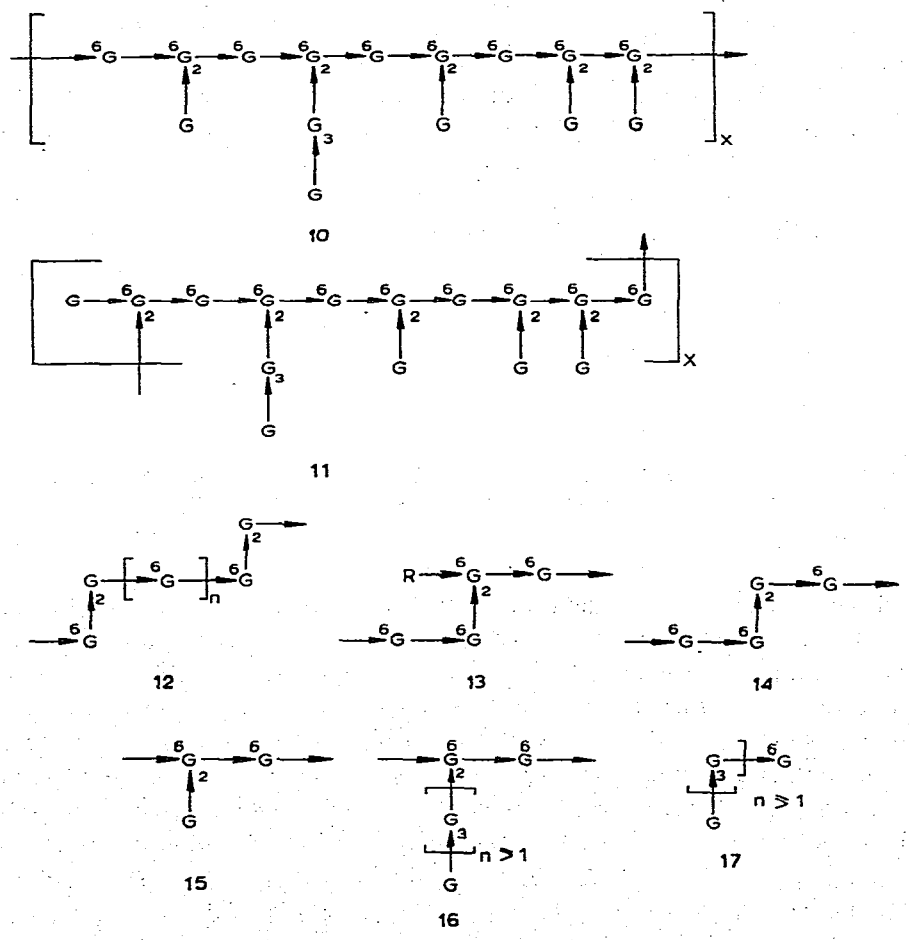

Fig. 2. Segments of dextran molecules. G, α-D-glucopyranosyl unit; R, α-D-glucopyranosyl unit or glycosyl units of isomaltose and its homologues.

(1→2)-*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→6)-D-glucose (**3**) and *O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→6)-D-glucose (**4**).

Structure of the dextrans. — Dextran *S* may possess two general types of structural features, consistent with the earlier reported[1] percentages of the variously linked D-glucose residues. The polymeric chain may continue either through α-(1→6)- or α-(1→2)-linkages, as exemplified by the average repeating-units **10** and **11** (Fig. 2), respectively. Some α-(1→3)-linkages in dextran *S* may[1,9,11] represent terminal linkages on non-reducing chain ends, as, for example, in **10** and **11**, although others may intersect a continuous chain of α-(1→6)-linked D-glucose residues. It is appreciated that some branches in both types of structure (**10** and **11**) may consist of more D-glucose residues than indicated. However, in view[1] of the high content (34%) of branching D-glucose residues in dextran *S* (*i.e.*, those linked through positions 1, 2, and 6), many must consist of only a few or, indeed, single D-glucose residues.

The results of the periodate oxidation of dextran *S-AD* show that many α-(1→2)- and all the α-(1→3)-linkages in dextran *S* had been hydrolysed when it was treated with acid. This is in agreement with the relative rates of hydrolysis of *O*-α-D-glucopyranosyl-D-glucoses[16–18]. The fact that the tetra- and penta-saccharides *A*, obtained from dextran *S-AD* by enzymic hydrolysis, did not contain components possessing α-(1→3)-linkages is further evidence that dextran *S-AD* did not possess α-(1→3)-linkages.

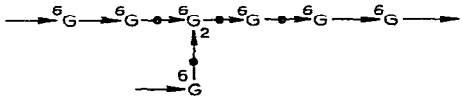

Fig. 3. Segment of dextran molecule showing glucosidic linkages which, if present, are resistant to hydrolysis by dextranase A. G, α-D-glucopyranosyl unit; —●→, glucosidic linkage resistant to hydrolysis.

Fig. 3 shows a segment of a dextran molecule in the vicinity of a D-glucose residue linked through a secondary linkage, and indicates the glucosidic linkages which, if present at all, are resistant[2,4,14] to hydrolysis by dextranase A. According to this action pattern, the unbranched oligosaccharides **1**–**4** must have been produced from unbranched sections of dextran *S-AD*. In addition, the reducing D-glucose residue in tetrasaccharide **2** must also represent the reducing D-glucose residue of dextran *S-AD*. The fact that the branched pentasaccharide *O*-α-D-glucopyranosyl-(1→6)-*O*-[α-D-glucopyranosyl-(1→2)]-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→6)-D-glucose (**9**) could not be detected when dextranase A had acted on dextran *S-AD* shows that such segments as that shown in Fig. 3 do not contribute significantly, if at all, to the structure of dextran *S-AD*. We thus conclude that the structure of dextran *S-AD*, in sections which can be degraded by dextranase A, is

essentially as represented by segment **12***. The structure of the oligosaccharides **1**, **3**, and **4**, together with the action pattern of dextranase A, further establish that the isomaltose homologues in segment **12**, which are mutually joined through positions 1 and 2 of their terminal D-glucose residues, have d.p. $\geqslant 4$, *i.e.*, in segment **12** $n \geqslant 2$.

In dextran *S*, all D-glucose residues linked through position 2 are also linked through position 6 and, hence, constitute branching residues. Therefore, the partial degradation of dextran *S* with acid to give dextran *S-AD* entailed debranching also by hydrolysis of the α-(1→6)-linkage on the non-reducing side of a branching D-glucose residue, *i.e.*, **13**→**14**.

It has been reported that acidic hydrolysis of dextran[6] (and of other glucans[19]) proceeds primarily by removal of small fragments (*i.e.*, D-glucose and small oligosaccharides) from non-reducing chain ends. The pronounced susceptibility to acidic hydrolysis of the α-(1→6)-linkage on the non-reducing side of some branching D-glucose residues in dextran *S* suggests, therefore, that these branching residues occur close to non-reducing ends of chains of α-(1→6)-linked D-glucose residues. As the majority of the "external" branches in dextran *S* appear to be single D-glucopyranosyl groups[6], we conclude that R in structure **13** also is most frequently an α-D-glucopyranosyl group (*cf.* the example **11** of an average repeating unit).

Kabat and co-workers[8,9,11] concluded, from immunochemical studies, that many terminal linkages in dextran *S* are of the α-(1→2) type. Sequences of α-(1→2)-linked D-glucose residues seem not to occur in dextran *S*, as the corresponding oligosaccharides [*e.g.*, *O*-α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl-(1→2)-D-glucose] could not be detected as products of acetolysis[14]. These observations, together with the fact that all D-glucose residues in dextran *S* which are linked through position 2 are branching residues[1], indicate that a high proportion of branches in dextran *S* are (1→2)-linked α-D-glucopyranosyl groups (as in segment **15**).

Present evidence (*cf.* Ref. 1) does not exclude the possibility that some of the α-(1→3)-linkages in dextran *S* intersect chains of α-(1→6)-linked D-glucose residues. However, the results of immunochemical studies[9–11] suggest that many of the α-(1→3)-linkages occur as terminal linkages on non-reducing chain ends. They are thus likely to occur at such chain ends as represented by segments **16** and/or **17**.

We now conclude that the polymeric chain in dextran *S* consists, principally, of segments of isomaltose homologues which are mutually linked through positions 1 and 2 of their terminal D-glucose residues. The average repeating-unit, containing a total of fifteen D-glucose residues, and of which structure **11** is one example, possesses five branches. These occur at each position 6 of such segments and at positions 2.

*[With Keith Marshall.] When we reached this conclusion, no more dextran *S-AD* was available. However, methylation analyses (*i.e.*, methylation, hydrolysis, reduction, acetylation, and g.l.c.–m.s.) of a sample of acid-degraded (hydrolysis for only 1.25 h) dextran *S* revealed, in addition to those D-glucose residues shown to be present in the native dextran *S*, D-glucose residues linked through positions 1 and 2 only. (The mass spectrum of the appropriate fraction showed a peak corresponding to *m/e* 189.) The results could not be evaluated quantitatively as, under the conditions used, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol and 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-glucitol could not be resolved by g.l.c.

Branches consist mainly of α-D-glucopyranosyl groups and some appear to be terminated by α-nigerosyl groups.

EXPERIMENTAL

Paper chromatography. — The solvent used with Whatman No. 1 paper was ethyl acetate–pyridine–water (2:1:2, organic phase). Migration rates are expressed relative to the movement of D-glucose (R_G).

Paper electrophoresis. — The electrolytes used were those described[20] in Part VI.

Spray reagents. — The spray reagents used were (*a*) and (*b*), described[21] in Part IX.

Gas–liquid chromatography. — This was carried out as described by Aspinall[22], using columns containing (*a*) butane-1,4-diol succinate polyester or (*b*) polyphenyl ether [*m*-bis-(*m*-phenoxyphenoxy)benzene]. Retention times [*T* (*column*)] are expressed relative to that of methyl 2,3,4,6-tetra-*O*-methyl-β-D-glucopyranoside.

Dextran. — The dextran fraction (dextran *S*) used was elaborated by *L. mesenteroides* NRRL B-1299 and has been described[1] in Part X.

Dextranases. — The dextranase preparations of *P. lilacinum* (C.M.I. 79197; NRRL 896) (dextranase A) and *P. funiculosum* (C.M.I. 79195; NRRL 1132) (dextranase B) used in the present work were prepared as described previously[5].

Preparation of dextran S-AD. — Dextran *S* (200 g) was hydrolysed with 0.5M sulphuric acid (5 l) at 90° for 1.75 h. The hydrolysate was neutralised with barium carbonate and filtered. The filtrate was concentrated *in vacuo* at 40° to *ca.* 1 litre, filtered, and dialysed for 3 days against running tap water using Visking Cellulose Tubing. The dialysed solution was concentrated further, as described above, and freeze-dried to give dextran *S-AD* (55 g, zero ash content).

Periodate oxidation. — The method used was as described before[12]. Dextran *S-AD* reduced 1.91 mol. of periodate with concomitant formation of 0.91 mol. of formic acid per "anhydroglucose" unit. The results of periodate oxidation of oligosaccharides (see below) are shown in Table II. Under the conditions used, isomaltose reduced 6.1 mol. of periodate with concomitant formation of 5.0 mol. of formic acid.

Degradation of dextran S-AD *by dextranase A to oligosaccharides.* — Dextran *S-AD* (50 g) in 0.2M sodium citrate (pH 5; 500 ml) was incubated with dextranase A (60 units contained in 500 ml of 5mM sodium citrate, pH 6) at 37° for 6 days. Paper chromatography of the deionised solution revealed the components shown in Table I. These, except the heptasaccharide, were isolated by methods similar to those described earlier[3,4]. Properties of the saccharides are shown in Tables I and II.

Methylation analysis of tetra- and pentasaccharide A. — Dry silver oxide (1 g) and redistilled methyl iodide (3 ml) were added to separate solutions of each oligosaccharide (~12 mg) in *N,N*-dimethylformamide (1.5 ml, redistilled from calcium oxide). Each mixture was shaken at room temperature in a darkened, sealed flask for 24 h. Solid materials were filtered off and washed with chloroform, and the filtrates

and washings were concentrated under diminished pressure at 40°. The residues were again treated by the above procedure. Traces of *N,N*-dimethylformamide were removed under diminished pressure at 30°. Each residue was dissolved in methyl iodide (5 ml), dry silver oxide was added, and the mixture was refluxed for 6 h. Solid materials were removed as before. The residue obtained from each combined filtrate and washings was dissolved in 4% methanolic hydrogen chloride (4 ml), the solution was refluxed for 5 h, neutralised with silver carbonate, filtered, and concentrated. G.l.c. of the syrupy residue obtained from each oligosaccharide revealed components having retention times identical with those of methyl α- and β-pyranosides of 2,3,4,6-tetra-*O*-[*T* (*a*) 1.00 and 1.43, *T* (*b*) 1.00 and 1.33], 2,3,4-tri-*O*-[*T* (*a*) 2.50 and 3.50, *T* (*b*) 1.33 and 1.83], and 3,4,6-tri-*O*-methyl-D-glucose [*T* (*a*) 2.93 and 3.50, *T* (*b*) 1.70 and 2.30].

Acetolysis of tetra- and penta-saccharide A. — Each oligosaccharide (~90 mg) was dissolved (by warming) in a mixture (3 ml) of acetic anhydride and sulphuric acid (100:9; v/v). Each solution was kept at 35° for 2 h and then poured onto crushed ice (10 g). Each mixture was adjusted to pH 6 by addition of sodium hydrogen carbonate, and the precipitated material was extracted with chloroform (4 × 10 ml). The extracts were washed with dilute, aqueous sodium hydrogen carbonate and water, dried (Na_2SO_4), and evaporated *in vacuo* at 40°. Each residue was dissolved in dry methanol (1 ml), and deacetylation was effected by addition of a small piece of sodium. After 24 h, each solution was deionised and concentrated, and the residue was fractionated by paper chromatography. Each fraction was subjected to electrophoresis in borate solution and, after treatment with sodium borohydride followed by deionisation, in molybdate solution. The results are shown in Table III.

Acidic and enzymic degradation of pentasaccharide A. — The oligosaccharide (~50 mg) was heated in 0.5M sulphuric acid (4 ml), in a capped tube, at 70° for 2.5 h. The hydrolysate was neutralised with barium carbonate, deionised, and fractionated by paper chromatography. The component (~5 mg) having R_G identical with that of isomaltotetraose was dissolved in 0.2M sodium citrate (pH 5, 2 ml) and incubated with dextranase A (0.24 unit contained in 2 ml of 5mM sodium citrate, pH 6) at 37° for 2 days. Paper chromatography of the deionised solution revealed that approximately half of the material had been degraded to the same compounds as was isomaltotetraose in a control experiment, *i.e.*, D-glucose, isomaltose (main product), and isomaltotriose.

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