STUDIES ON DEXTRANS AND DEXTRANASES

PART IX[1]. DEXTRANS ELABORATED BY CARIOGENIC ORGANISMS

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(Received December 4th, 1970; accepted for publication, January 25th, 1971)

ABSTRACT

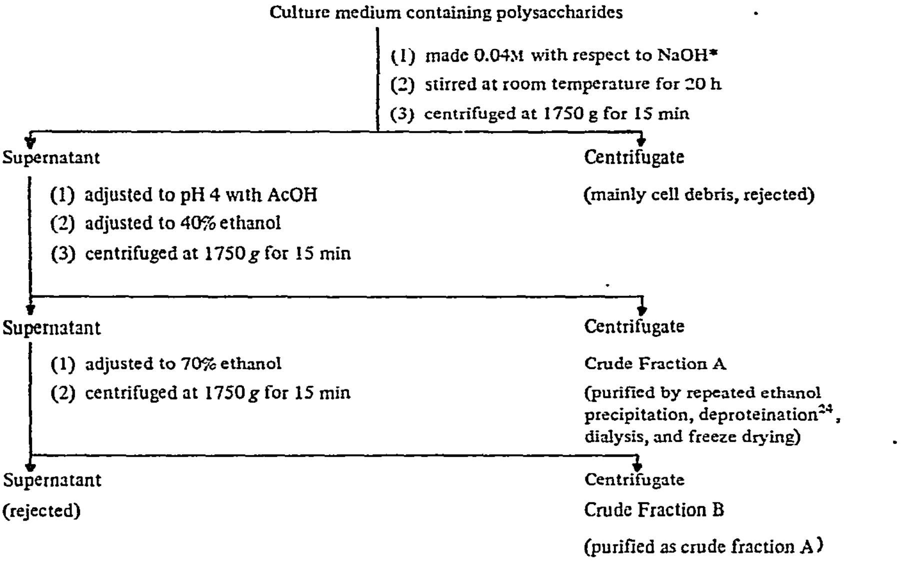
The cariogenic *Streptococcus* Ingbritt and *S.* OMZ 51 and the non-cariogenic *S.* ATCC 10558 produce polysaccharides, the major component of which is of the dextran type. A high proportion of the glucose residues in the dextran-like materials are linked through non-α-D-(1 → 6) linkages. The number of "anhydro-glucose" units in the average repeating-units of these materials ranges from 4 to 11. It is likely that the non-α-D-(1 → 6) linkages form branch points. The methods used failed to reveal significant structural differences between the dextran-like materials produced by cariogenic and non-cariogenic organisms.

INTRODUCTION

Several strains of streptococci, described as cariogenic in rodents and/or man, produce extracellular polysaccharides when grown in sucrose-containing media[2]. The suggestion that some of these are dextrans stems mainly from the observations that (*a*) glycerol is detectable (by paper chromatography) in the products of a Smith degradation of the polysaccharides[3–5], (*b*) the polysaccharides seem to be degraded by dextranase[6,7], (*c*) the polysaccharide appeared to be antigenically identical with the dextran elaborated by a strain of *Leuconostoc mesenteroides*[6], and (*d*) the infrared spectra contained absorption peaks which are also contained in those of a dextran[5]. The facts that glucose and erythritol were minor components in the products of Smith degradations and that the polysaccharides exhibited diffuse absorption at 793 cm^{-1} have been presented as evidence for the existence of branch linkages[5]. It is also known[2] that several cariogenic strains of streptococci produce considerable amounts of iodophilic polysaccharides when grown in media containing glucose, maltose, or sucrose. It thus seemed desirable to study in greater detail the chemical structure of polysaccharides associated with dental caries. For our initial survey, we have selected the polysaccharides elaborated by two cariogenic streptococcal strains, namely Ingbritt and OMZ 51. These micro-organisms were originally isolated from carious lesions[8] in humans and were shown to be cariogenic in hamsters[8] and monkeys[9] (Ingbritt) and rats[10] (OMZ 51). The results are also compared with those obtained from the polysaccharide material elaborated by the strain ATCC 10558, which is non-cariogenic in the rat[11].

RESULTS AND DISCUSSION

Two polysaccharide fractions (*A* and *B*) were isolated from the culture media of each organism by precipitation from 40 and 70% ethanol, respectively (see Fig. 1); yields and some properties are shown in Table I. Fraction ATCC 10558(*A*) was considerably less-soluble in water than the other materials.



*Culture medium of ATCC 10558 was made 0.1M.

Fig. 1. Isolation of polysaccharide fractions.

Paper chromatography of acid hydrolysates of each polysaccharide fraction revealed glucose and fructose as the only monosaccharide components. Determination of the glucose and fructose content of the fractions showed (Table I) that the polysaccharide material produced by each organism is not homogeneous and that the precipitation procedure had effected a partial separation of glucan from fructan or glucofructan components, fractions *A* being mainly glucans. No further attempts to obtain homogeneous polysaccharide materials have been made. It is thus emphasised that the structural features mentioned in the ensuing discussion are not necessarily features of homogeneous polysaccharides.

Partial, acid hydrolysates and partial acetolysates of each polysaccharide fraction were shown by paper chromatography to contain the oligosaccharides of the isomaltose series. This indicates that the polysaccharide materials contain components

TABLE I

GENERAL PROPERTIES OF POLYSACCHARIDE FRACTIONS

Polysaccharide fraction	*Yield (g) from 150 g of sucrose*	*Ash (%)*	*N (%)*	$[\alpha]_{5461}$[a] *(degrees)*	*Glucan content*[b] *(%)*	*Fructan content*[c] *(%)*
Ingbritt(*A*)	1.10	2.07	1.16	+232	97.4	5.9
Ingbritt(*B*)	0.60	5.51	1.28	+6	34.9	57.9
OMZ 51(*A*)	3.00	1.88	1.02	+236	98.6	1.4
OMZ 51(*B*)	0.70	3.03	1.16	+261	94.7	2.6
ATCC 10558(*A*)	1.63	3.95	1.04	+279	98.3	0.1
ATCC 10558(*B*)	0.34	6.86	1.31	+233	75.0	16.9

[a] Concentrations varied between 0.1 and 0.3% in water. [b] Calculated as if glucose liberated on hydrolysis originated only from glucan. [c] Calculated as if fructose liberated on hydrolysis originated only from fructan.

possessing continuous chains of α-(1→6)-linked-D-glucopyranose units, *i.e.* the building blocks of dextran. This is in agreement with the fact that each fraction was extensively degraded by dextranase (see also below), but not by alpha-amylase.

The controlled degradation of selected fractions (*i.e.* fractions containing little fructose) by periodate oxidation, reduction, and hydrolysis gave products from which it was concluded that, in these fractions, glucose residues are linked by the (1→2)-linkage (glyceraldehyde and glycerol), (1→3)-linkage (glucose), (1→4)-linkage (only to a small extent; glycolaldehyde and erythritol), as well as the (1→6)-linkage (glycolaldehyde and glycerol). These results are in agreement with those obtained from the partial acetolysis of all fractions, which revealed that kojibiose, nigerose, and maltose [only for OMZ 51(*A*), Ingbritt(*A*), Ingbritt(*B*)] represent structural segments of these polysaccharides.

The percentages of the variously linked glucose residues in the selected fractions, calculated from the amount of periodate reduced and formic acid produced on treatment with periodate, are shown in Table II. The fact that this evaluation, in contrast to the results of the controlled degradation and acetolysis, did not reveal the presence of (1→3)-linked-glucose residues in the OMZ 51(*B*) fraction emphasises that such results, when in isolation, have to be interpreted cautiously.

The n.m.r. spectra of the deuterated fractions OMZ 51(*A*), OMZ 51(*B*), and Ingbritt(*A*) [fraction ATCC 10558(*A*) was insufficiently soluble in D_2O for n.m.r. measurements] contained signals at τ 5.04 and 4.68 which arise[12,13] from the protons on the anomeric carbon atoms involved in α-D-(1→6) linkages and non-α-D-(1→6) linkages, respectively. [The spectra also contained signals at τ 5.25 (DOH in solvent used), 6.20 (H-2, H-3, H-4), and 6.41 (H-5 and H-6)]. From the area under these peaks, the percentages of glucose residues linked through α-D-(1→6) and non-α-D-(1→6) linkages have been calculated (see Table II). The results are in reasonable agreement with those obtained from periodate-oxidation experiments. From the results of the periodate oxidations, the numbers of "anhydro-glucose" units in the average repeating-units have been calculated (see Table II).

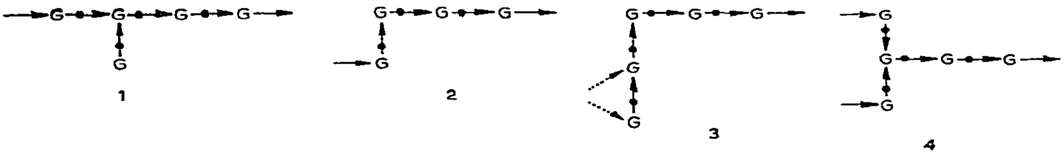
TABLE II

PERCENTAGE OF VARIOUSLY LINKED GLUCOSE RESIDUES

Polysaccharide fraction	*Periodate oxidation*						*N.m.r. data*		
	Periodate reduced^b	*Formic acid produced*^{b}	*"Anhydro-glucose" units linked through*			*No. of "anhydro glucose" units in average repeating-unit*	*Ratio of areas under peaks at* τ *5.04 and 4.68*	*"Anhydro-glucose" unit linked through*	
			(1→6) only (%)	*(1→2)-like* (%)	*(1→3)-like* (%)			*(1→6)* (%)	*non-(1→6)* (%)
Ingbritt(*A*)	1.51	0.67	67	17	16	6	2.6	72	28
OMZ 51(*A*)	1.63	0.77	77	9	14	11	3.8	79	21
OMZ 51(*B*)	1.76	0.76	76	24	0	4	4.9	83	17
ATCC 10558(*A*)^a	1.59	0.68	68	23	9	11			

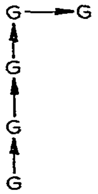
[a] Insufficiently soluble in D_2O for satisfactory n.m.r. spectrum. [b] Moles per mole of "anhydro-glucose" unit.

In the dextrans studied in detail[1], the non-α-D-(1 → 6) linkages are usually branch linkages on a continuous chain of α-D-(1 → 6)-linked-glucose residues (*e.g.* **1**) but are also reported[13] to occur as non-branching linkages (*e.g.* **2**). The above methods do not distinguish between these structures, and the technique of methylation is often used for this purpose. However, it was conceived that some knowledge of structural features in the vicinity of the non-α-D-(1 → 6) linkages might be gained from the pattern of products of enzymic hydrolysis. The dextranase of *Penicillium lilacinum* (I. M. I. 79197; NRRL 896) (dextranase A of Part II[14]) was chosen for this investigation. The smallest homologue of isomaltose readily hydrolysed by dextranase A is isomaltotetraose, isomaltose being the main product. Segments of dextran molecules showing the glucosidic linkages in the vicinity of non-α-D-(1 → 6) linkages reported[13,15] not to be hydrolysed by dextranase A are depicted by structures **1** and **2**. Thus, the smallest oligosaccharide containing the non-(1 → 6)-linkage obtained from the branch structure **1** is a pentasaccharide, whereas that from structure **2** is a tetrasaccharide.



Segments of dextran molecules in the vicinity of non-(1→6)-linkages. G, D-glucopyranosyl unit; →, α-D-(1→6) linkage which can be hydrolysed by dextranase A; ⇢ alternative α-D-(1→6) linkage which can be hydrolysed by dextranase A; -●►, α-D-(1→6) linkage resistant to hydrolysis by dextranase A; ↕ or ↕, non-(1→6)-linkage resistant to hydrolysis by dextranase A.

Each polysaccharide fraction was extensively degraded by dextranase A. The products (in addition to glucose, isomaltose, isomaltotriose, and unresolvable material) had properties similar to the branched penta-, hexa-, hepta-, and octasaccharides obtained by the action of dextranase A on branched dextrans[13,16,17]. The differences between the chromatographic migration rates of the series of oligosaccharides possessing α-D-(1 → 2), α-D-(1 → 3), or α-D-(1 → 4) branch-linkages are too small for a more precise assignment. A tetrasaccharide could not be detected in the reaction mixtures. These results show that the segment **2** does not contribute greatly, if at all, to the structure of the polysaccharide fractions. According to the mechanism of the action[15] of dextranase A, the pentasaccharides containing fewer than two α-(1 → 6)-linked-D-glucose residues (*e.g.* **5**) and the pentasaccharide **6** can only arise from reducing and non-reducing chain-ends, respectively. It is thus likely that the polysaccharides possess segments **1**, **3**, and/or **4**, giving rise, on treatment with dextranase, to the corresponding pentasaccharides. It is noteworthy that structural features represented by **3** and **4** have, as yet, not been detected in dextrans.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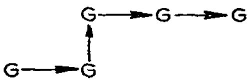
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Pentasaccharides which could arise from chain ends. G, D-glucopyranosyl unit; →, α-D-(1→6) linkage; ↑, non-(1→6)-linkage.

Previously, it has been noted[2] that cariogenic streptococci characteristically produce relatively large amounts of glucose-containing polysaccharides, whereas the polysaccharides produced by non-cariogenic strains were primarily of the fructose-containing type. Table I (percent fructan of total isolated polysaccharide) shows that the polysaccharides of the strains ATCC 10558 and Ingbritt are exceptions to this generalisation.

It has also been suggested[7] that the dextrans produced by cariogenic strains are more highly branched than those produced by non-cariogenic strains. Although chemical evidence for the existence of branching glucose residues in the dextrans elaborated by cariogenic strains has, as yet, not been presented, the percentage of non-α-(1→6)-linked-D-glucose residues and the sizes of the repeating units of the four fractions examined by us in some detail do not indicate that dextrans elaborated by cariogenic organisms are distinguishable by these properties.

The observations[2] that dextrans form insoluble precipitates with salivary proteins and may be adsorbed on hydroxyapatite, and that dextran-producing organisms have been identified in human plaque material suggested that dextran formation by micro-organisms may be an important factor in the formation of dental plaques and, by extension, caries under conditions of excessive intake of sucrose. The methods used in this initial survey failed to reveal significant, structural differences between the dextran-like fractions produced by cariogenic and non-cariogenic organisms. Clearly, more-detailed studies, notably including the use of more-refined methods of polysaccharide separation and purification, are needed before a common chemical property, if it exists at all, can be assigned to dextrans involved in the formation of dental plaques.

EXPERIMENTAL

General. — Optical rotations were determined on aqueous solutions at room temperature, using a Hilger–Watts microptic, photoelectric polarimeter. Ash and nitrogen contents were determined by A. Bernhardt, Munich. The solvents used for paper chromatography were (*a*) ethyl acetate–acetic acid–formic acid-water (18:3:1:4); (*b*) ethyl acetate–pyridine–water (2:1:2, organic phase); (*c*) butyl alcohol-pyridine–water (6:4:3); (*d*) butyl alcohol–ethanol–water (40:11:19). The electrolytes used for paper electrophoresis were (*a*) 0.2M sodium borate[18]; (*b*) 2% sodium molybdate[19] adjusted to pH 5. The spray reagents used for the detection of compounds were (*a*) silver nitrate in acetone–ethanolic sodium hydroxide[20]; (*b*) urea–phosphoric acid[21]; (*c*) aniline–diphenylamine–phosphoric acid[22].

Preparation and purification of polysaccharides. — The polysaccharides examined were produced by *Streptococcus* OMZ 51, *S.* Ingbritt, and *S.* ATCC 10558. The procedure was as follows. An 18-h culture (1 ml) of each organism was used to inoculate separate culture media (300 ml) contained in dialysis sacs. The medium contained tryptone (Oxoid) (1%), yeast extract (0.5%), K_2HPO_4 (0.3%), and sucrose (5%). Each dialysis sac was immersed in medium (3 l, composition as above) contained in flasks which were sealed with plastic bags and the latter flushed with carbon dioxide. The cultures were incubated at 37° for 72 h. The polysaccharide materials were isolated from the culture media and fractionated by the scheme outlined in Fig. 1. The yields of the fractions obtained and some of their properties are shown in Table I.

Acid hydrolysis of the polysaccharides. — (*i*) Each polysaccharide fraction (50 mg) was separately hydrolysed with 0.1M sulphuric acid (2 ml) for 1 h at 70°. Paper chromatography [solvent (*a*)] of each hydrolysate (after neutralisation with aqueous barium hydroxide and filtration) revealed, as the main component, a material which had migration and staining properties identical with those of fructose. A trace component in each hydrolysate had properties similar to those of glucose.

The fructose liberated from each polysaccharide fraction was determined by the alcoholic anthrone method[21]. An acid-degraded preparation of the dextran elaborated by *Leuconostoc mesenteroides* NRRL B-512 was used as a control. The results, expressed as fructan content and shown in Table I, were obtained by using the equations of Wise *et al.*[21], but incorporating an allowance for the independently determined glucan content of the test samples, *i.e.* weight of fructose = K (A unknown − A control × G/100), where G is the glucan content of the test sample.

(ii) The polysaccharide fractions (20 mg) were separately hydrolysed with M sulphuric acid (2 ml) for 7 h at 100°. Paper chromatography, using solvent (*a*), and paper electrophoresis (in borate solution) of each deionised (see above) hydrolysate revealed, as the main component, a material which had migration and staining properties identical with those of glucose. A trace component in the hydrolysates of fractions Ingbritt(*B*) and ATCC 10558(*B*) had properties similar to those of fructose.

Each deionised hydrolysate was treated with potassium borohydride. Paper electrophoresis revealed, in each case, a material, as main component, which had migration and staining properties identical with those of glucitol.

After hydrolysis of another sample of each polysaccharide fraction (50 mg, neutralisation was by addition of an equal volume of 2M sodium hydroxide), the amount of D-glucose liberated was determined with the D-glucose oxidase–peroxidase reagent (Boehringer Biochemicals). The results, expressed as glucan content, corrected for ash and glucose degraded under the above conditions[23], are shown in Table I.

(iii) Each polysaccharide fraction (*ca.* 30 mg) was partially hydrolysed with 0.5M sulphuric acid (3 ml) for 1.5 h at 100°. Paper chromatography, using solvent (*b*), of each deionised hydrolysate revealed the presence of components which had migration rates and staining properties identical with those of glucose and the oligosaccharides of the isomaltose series.

Degradation of polysaccharides by dextranase. — Each polysaccharide fraction (*ca.* 10 mg) was treated separately with the dextranase preparation (1 mg) of *Penicillium lilacinum*[14] (I. M. I. 79197; NRRL 896) in a manner similar to that described earlier[16]. The digests were deionised with Amberlite IR-120(H^+) and IRA-400(OH^-) resins. Paper chromatography, using solvent (*b*), revealed in each case components which had migration rates and staining properties similar to those of the products of the action of the above dextranase on the dextrans elaborated by *Leuconostoc mesenteroides* NRRL B-1375 (Birmingham)[16], NRRL B-1415[17], and NRRL B-1299[13].

Treatment of polysaccharides with alpha-amylase. — Separate solutions of each polysaccharide fraction and soluble starch (BDH reagent) (*ca.* 10 mg) in 15mM sodium β-glycerophosphate (BDH reagent) (adjusted to pH 5.7, 3 ml) were incubated with alpha-amylase preparation (2 mg) of *Bacillus subtilis* (Koch–Light reagent) for 72 h at 35°. Paper chromatography, using solvent (*b*), revealed products of degradation only in the digest containing soluble starch.

Acetolysis. — The polysaccharide fractions were partially acetolysed in the following manner. The polysaccharide (*ca.* 100 mg) was heated with a mixture (4 ml) of acetic anhydride and concentrated sulphuric acid (100:9, v/v) at 35° for 30 h. The mixture was then poured onto crushed ice (*ca.* 15 g), neutralised by addition of solid sodium hydrogen carbonate, and extracted with chloroform (3 × 15 ml). The chloroform extract was washed with saturated, aqueous sodium hydrogen carbonate and water, dried (Na_2SO_4), and concentrated. The syrupy residue was dissolved in absolute methanol (15 ml), and deacetylation was effected by addition of a small piece of sodium. After 2 days, distilled water (*ca.* 5 ml) was added, and the solution was deionised by treatment with Amberlite IR-120(H^+) and IRA-400(OH^-) resins and evaporated. The syrupy residue was fractionated by paper chromatography, using solvent (*c*). Each isolated component was analysed by paper electrophoresis in borate solution and, after treatment with potassium borohydride, in molybdate solution. (For results, see RESULTS AND DISCUSSION).

Periodate Oxidation. — The selected polysaccharide fractions (*ca.* 150 mg, dried *in vacuo* over phosphoric oxide) were separately oxidised with 10mM sodium metaperiodate (250 ml) in the dark at room temperature. At intervals, portions were withdrawn for the determination of periodate reduced and formic acid produced by titration with arsenite and sodium hydroxide solutions, respectively. The values became constant after *ca.* 100 h. The results are shown in Table II.

Fragmentation of periodate-oxidised polysaccharides. — The selected polysaccharide fractions (*ca.* 100 mg) were separately oxidised with 50mM sodium metaperiodate (25 ml) in the dark at room temperature for 5 days. Ethylene glycol (*ca.* 0.5 ml) was added to each solution which was then dialysed against running water (3 days). Sodium borohydride (75 mg) was added to each solution which was again dialysed

(3 days). The polyalcohols were obtained as freeze-dried, white powders: ATCC 10558(*A*) (87 mg); OMZ 51(*A*) (73 mg); OMZ 51(*B*) (51 mg); Ingbritt(*A*) (65 mg).

The polyalcohols (*ca.* 10 mg) were separately hydrolysed with 0.5M sulphuric acid (2 ml) at 100° for 5 h. Paper chromatography and paper electrophoresis in borate solution revealed each deionised hydrolysate to contain components which had migration and staining properties identical with those of glucose, glycolaldehyde, glycerol, glyceraldehyde (trace), and erythritol (trace).

Nuclear magnetic resonance spectra. — The selected polysaccharide fractions (*ca.* 80 mg) were thrice freeze-dried from deuterium oxide (2 ml). The n.m.r. spectra of the deuterated polysaccharides were recorded with a Varian IL 60 HA instrument. Sodium 4,4-dimethyl-4-silapentane-1-sulphonate was used as internal standard for peak assignments. The ratios of the peaks at τ 5.04 and 4.68 are shown in Table II.

ACKNOWLEDGMENTS

The authors thank Professor E. J. Bourne for valuable discussions and the Science Research Council for financial support.

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