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CHARACTERIZATION OF THE EXTRACELLULAR, WATER-INSOLUBLE α-D-GLUCANS OF ORAL STREPTOCOCCI BY METHYLATION ANALYSIS, AND BY ENZYMIC SYNTHESIS AND DEGRADATION*†

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ABSTRACT

Methylation analysis of water-insoluble α-D-glucans synthesized from sucrose by culture filtrates from several strains of *Streptococcus* spp. has proved that all of the glucans were highly branched and that the chains contained (1→6)- and (1→3)-linked D-glucose residues not involved in branch points. Hydrolysis of the glucans with a specific endo-(1→3)-α-D-glucanase demonstrated that the majority of the (1→3)-linked glucose residues were arranged in sequences. D-Glucose was the major product of the hydrolysis, and a small proportion of nigerose was also released. The use of a specific endo-(1→6)-α-D-glucanase similarly indicated that the glucans also contained sequences of (1→6)-linked α-D-glucose residues, and that those chains were branched. Two D-glucosyltransferases (GTF-S and GTF-I), which reacted with sucrose to synthesize a soluble glucan and a water-insoluble glucan, respectively, were separated from culture filtrates of *S. mutans* OMZ176. The soluble glucan was characterized as a branched (1→6)-α-D-glucan, whereas the insoluble one was a relatively linear (1→3)-α-D-glucan. The hypothesis is advanced that the glucosyltransferases can transfer glucan sequences by means of acceptor reactions similar to those proposed by Robyt *et al.* for dextransucrase, leading to the synthesis of a highly branched glucan containing both types of chain. The resulting structure is consistent with the evidence obtained from methylation analysis and enzymic degradations, and explains the synergy displayed when the two D-glucosyltransferases interact with sucrose. Variations in one basic structure can account for the characteristics of water-insoluble glucans from *S. sanguis* and *S. salivarius*, and for the strain-dependent diversity of *S. mutans* glucans.

*Dedicated to Dr. Allene Jeanes on the occasion of her retirement.

†Part III of the series Metabolism of the Polysaccharides of Human Dental Plaque.

INTRODUCTION

Most native dextrans synthesized from sucrose by various strains of *Leuconostoc* spp. contain a proportion of (1→3)-α-D-glucosidic linkages[1,2]. These generally occur at branch points, whereas (1→6)-α-D-glucosidic linkages predominate in the main chains, By contrast, methylation studies[3–7] have indicated that (1→3)-α-D-glucosidic linkages may be found both at branch points and in the main chains of the extracellular α-D-glucans that are synthesized from sucrose by oral streptococci. A D-glucosyltransferase (GTF-I) specific for the synthesis of water-insoluble (1→3)-α-D-glucan has been isolated from culture filtrates of *Streptococcus mutans* OMZ176 (refs. 8, 9) and K1-R (ref. 10).

The ability to synthesize water-insoluble extracellular glucans is directly related to the establishment[11] and cariogenicity[12] of *S. mutans* in dental plaque. Binding of the glucans to hydroxylapatite[13] and to acceptor proteins on the cell surface[14] constitutes a mechanism for the adherence of *S. mutans* to tooth surfaces. The diffusion barrier created by insoluble glucan may cause an accumulation of acid in plaque, and furthermore, the presence of insoluble polysaccharide is considered to aid the survival of *S. mutans* in acidic environments[15]. Mutant strains that are incapable of synthesizing insoluble glucan show decreased adherence, decreased cariogenicity and decreased powers of survival[16–18].

Support for the roles postulated for GTF-I and (1→3)-α-D-glucans in the events that lead to caries has been provided by the sequelae of administering (1→3)-α-D-glucanases into the oral cavity. A significant decrease in the proportion of *S. mutans* in human dental plaque followed a mouth-rinse with *Aspergillus nidulans* (1→3)-α-D-glucanase[19], and treatment of rats harbouring an indigenous cariogenic flora with *Trichoderma harzianum* mutanase[20], an enzymic preparation containing (1→3)-α-D-glucanase, resulted in a dramatic reduction in the incidence of caries[21]. Similar experiments with dextranase have not always been successful in caries reduction, and the conflicting results may arise from differences in the structure of the insoluble glucans that are produced in dental plaque by different infecting organisms. All serotypes of *S. mutans* produce a D-glucosyltransferase (GTF-S) that synthesizes a soluble, dextran-like glucan from sucrose[22]. Thus, the type of extracellular glucan produced by various streptococci will depend on the relative activities, action patterns, and interaction of the D-glucosyltransferases and dextranases that are released under the particular conditions of growth of each organism.

We have investigated the use of specific endo-[9,10,23] and exo-dextranases[24,25] for the hydrolysis of (1→6)-α-D-glucosidic linkages in α-D-glucans of oral streptococci. The endo-(1→3)-α-D-glucanase[9] of *Cladosporium resinae* and endodextranase (Calbiochem)[23] have been used, separately and in combination, to estimate the proportion of (1→3)- and (1→6)-linked sequences of D-glucose residues in several α-D-glucans. Methylation analysis of the same polysaccharides has confirmed and expanded the enzymic studies by providing quantitative information on the proportion of the individual linkages, and on the degree of branching. Our preliminary results on

the concerted action of the D-glucosyltransferases involved in the synthesis of the (1→6)- and (1→3)-linked D-glucan chains respectively, indicate how these enzymes may interact to synthesize insoluble glucan. A structure consistent with the combined data is proposed for the mixed-linkage α-D-glucans of *S. mutans* and *Streptococcus sanguis*.

MATERIALS AND METHODS

Bacteria. — All strains of streptococci were isolated from human dental plaque. Strains designated NSW were isolated by Dr. R. G. Schamschula from New South Wales school-children. Reference strains of *Streptococcus mutans* Ingbritt, B13, K1-R, and OMZ176 were kindly provided by Professor B. Krasse, Dr. S. Edwardsson, Dr. R. J. Fitzgerald, and Professor B. Guggenheim, respectively, and *Streptococcus sanguis* 804 (NCTC10904) was a gift from Professor J. Carlsson. The streptococci were grown overnight in a medium of D-glucose in a Microferm fermentor (New Brunswick Scientific Co.), with the pH controlled at 6.0, under the conditions described previously[26]. The bacteria were then removed by centrifuging for 10 min at 2° at 4,000*g*, giving a cell-free supernatant solution (CFF).

Carbohydrates. — α-D-Glucans of oral streptococci were prepared by incubating cell-free filtrates with sucrose (4%) for 20 h at 35° under toluene. The water-insoluble polysaccharides were collected by centrifuging for 15 min at 3,000*g*, washed (×8) with water, and freeze-dried. Analysis with anthrone reagent[27] showed that the products were 100% carbohydrate. (1→3)-α-D-Glucans of *S. mutans* were prepared similarly, by the action on sucrose of purified D-glucosyltransferase-I[9,10]. A soluble glucan was synthesized from sucrose with D-glucosyltransferase-S isolated from culture filtrates of *S. mutans* OMZ176 (ref. 9). The glucan was precipitated and washed with 66% ethanol, dissolved in water, reprecipitated and washed with 75% ethanol, dissolved in water, and freeze-dried.

Soluble dextrans synthesized by *Leuconostoc mesenteroides* strains NRRL B-512(F) and B-1355 were kindly provided by Dr. Allene Jeanes. Dextran 2000 (mol. wt. 2×10^6) was purchased from Sigma Chemical Co.; other dextran fractions having lower weight-average mol. wt. were kindly provided by Pharmachem. Corp. (Bethlehem, Pa., U.S.A.), and chemically synthesized[28] dextran, a linear (1→6)-α-D-glucan, was a gift from Professor C. Schuerch. [U-^{14}C]Sucrose was purchased from ICN Pharmaceuticals, Inc., Irvine, California. Sucrose was dialysed through cellulose casing before use. The concentration of sucrose and dextran solutions was determined with anthrone reagent[26].

Enzymes. — (1→3)-α-D-Glucanase (EC 3.2.1.59) was isolated from the culture fluids of *Cladosporium resinae* QM7998. The culture conditions and purification of the enzyme were described previously[9]. A bacterial endo-(1→6)-α-D-glucanase (EC 3.2.7.11) was isolated[23] from the product supplied by Calbiochem., Los Angeles (dextranase-CB), and a similar enzyme was prepared from *S. mutans*[9,10]. The (1→6)-α-D-glucan glucohydrolase (EC 3.2.1.70) was isolated[24] from *Streptococcus mitis* 439.

and the (1→6) α-D-glucan isomaltohydrolase[25] of *Arthrobacter globiformis* T6 was kindly provided by Professor T. Sawai. D-Glucosyltransferase-I (GTF-I) was isolated from *S. mutans* K1-R by the method of Pulkownik and Walker[10]. GTF-S and GTF-I were separated from culture filtrates of *S. mutans* OMZ176 as described by Walker and Hare[9].

Units. — The unit of activity of all of the D-glucanases is defined as the amount of enzyme that liberates 1 μmol of reducing sugar per min under standard conditions. One unit of D-glucosyltransferase activity is the amount of enzyme required to transfer 1 μmol of D-glucose from sucrose to glucan in one min.

Limit of enzymic hydrolysis and solubilization of water-insoluble α-D-glucans. — Each glucan (1 mg) was incubated for 5 days at 35° with enzyme in digests (0.3 ml) buffered to an appropriate pH. Insoluble material was then removed by centrifuging, and the concentration of glucose, reducing sugars, and soluble carbohydrate in the supernatant solution was determined as described previously[9]. Hydrolysis by endo-(1→6)-α-D-glucanases (0.022 unit) and isomaltohydrolase (0.18 unit) was expressed as apparent conversion into isomaltotriose and isomaltose, respectively, and hydrolysis by endo-(1→3)-α-D-glucanase (0.23 unit) and by exo-(1→6)-α-D-glucanase (0.18 unit) was reported as conversion into glucose. The soluble products were separated and examined by paper chromatography on Whatman No. 3MM paper with 10:4:3 ethyl acetate–pyridine–water. The sugars were detected with alkaline silver nitrate[29].

Determination of D-glucosyltransferase activity. — GTF-S was assayed at 35° by incubating the enzyme with sucrose (4%), sodium citrate buffer (pH 6.5, 20mM), and dextran (mol. wt. 18,000, 0.4 mg/ml). Portions (0.25 ml) were withdrawn at intervals of 0.5, 1, and 3 h, and treated with 3 vol. of ethanol. After being kept for over 1 h at 4°, the precipitated polysaccharide was recovered by centrifuging, washed twice with 75% ethanol (2 ml), and dissolved in water (0.5 ml). The weight of soluble glucan was determined with the anthrone reagent[27]. GTF-I was assayed similarly, except that the water-insoluble product was washed with water, dissolved in 7M sulphuric acid, and then determined with anthrone reagent[27].

Synthesis of soluble and water-insoluble glucan. — Comparisons between the weight of glucan synthesized by GTF-I and GTF-S, acting alone or together, were made under the same conditions as the assays of activity, except that dextran was omitted.

The effect of added polysaccharides (50 μg) on glucan synthesis was tested in assay mixtures (0.25 ml) containing, in addition, [^{14}C]sucrose (0.125 μCi, 30 μg). After incubation for 1 h, the labelled glucan was separated from sucrose by filtering through Whatman No. 3MM paper (25 mm diameter). The paper discs were washed 12 times with water (2 ml), and once with methanol (5 ml). The amount of water-insoluble glucan was then determined by counting in a toluene cocktail.

Methylation analysis. — α-D-Glucans synthesized with GTF-I, GTF-S, or unfractionated cell-free filtrates of oral streptococci were methylated and analysed as described by Björndal *et al.*[30].

RESULTS AND DISCUSSION

Analysis of (1→3)-α-D-glucosidic linkages. — Methylation analysis of the polysaccharides (Table I) revealed that extracellular D-glucosyltransferases from culture filtrates of *S. mutans* strains produced water-insoluble glucans containing between 38 and 64% of (1→3)-D-glucosidic linkages that were not involved in branching. The results are comparable with values of 2,4,6-tri-*O*-methylglucose reported previously for the analysis of water-insoluble glucans of *S. mutans* strains OMZ176 (51%)[3,5], Ingbritt (52%)[4], and JC2 (46%)[7]. Glucans synthesized with D-glucosyltransferase-I (GTF-I) preparations from *S. mutans* strains OMZ176 and K1-R gave 88 and 85%, respectively, of 2,4,6-tri-*O*-methylglucose. These values correlate well with the extensive hydrolysis and complete solubilization of the glucans by the (1→3)-α-D-glucanase of *C. resinae* (Table II), and confirm a previous report[9] that GTF-I converts sucrose into water-insoluble (1→3)-α-D-glucan. The methylation studies further indicated that the degree of branching of the (1→3)-α-D-glucans is low (~2% of 2,4-di-*O*-methylglucose), whereas glucans synthesized with unfractionated D-glucosyltransferase (CFF) from all of the streptococci are highly branched, giving, on average, 13% of 2,4-di- and 2,3,4,6-tetra-*O*-methylglucose (Table I).

TABLE I

METHYLATION ANALYSIS OF α-D-GLUCANS

Source of glucan[a]	*Methyl ethers (mol %)*			
	2,4,6-Tri-	*2,3,4-Tri-*	*2,3,4,6-Tetra-*	*2,4-Di*
S. mutans (GTF)				
K1-R GTF-I	85	4	9	2
OMZ176 GTF-I	88	7	4	1
OMZ176 GTF-S	trace	31	37	32
S. mutans (CFF)				
K1-R	64	11	12	13
OMZ176	57	18	15	10
B13	64	11	12	13
Ingbritt	53	36	7	4
NSW1	51	32	7	10
NSW47	38	39	12	11
NSW47(S)	28	41	15	16
S. salivarius (CFF)				
ATCC13419	59	14	13	15
S. sanguis (CFF)				
804	21	49	15	15
804(S)	9	61	15	15
ATCC10558(S)	17	47	18	18
NSW13	19	48	18	15
L. mesenteroides				
B-1355(S)	34	44	11	11

[a]S refers to soluble glucan; all other glucans were water-insoluble.

TABLE II

ENZYMIC HYDROLYSIS AND SOLUBILIZATION OF α-D-GLUCANS

Source of glucan	*Limit of hydrolysis and solubilization*[a] (%)		
	C. resinae (*1→3*)-α-D-*glucanase*	*Dextranase-CB*	(*1→3*)-α-D-*Glucanase plus dextranase*
S. mutans (GTF)			
K1-R GTF-I	92 (100)	4 (7)	96 (100)
OMZ176 GTF-I	84 (100)	4 (7)	96 (100)
OMZ176 GTF-S	0	15	22
S. mutans (CFF)			
K1-R	49 (54)	5 (21)	65 (94)
OMZ176	40 (51)	22 (46)	51 (92)
B13	61 (81)	3 (21)	68 (93)
Ingbritt	32 (42)	31 (42)	82 (92)
NSW1	34 (70)	38 (54)	79 (100)
NSW47	27 (36)	41 (63)	70 (100)
S. salivarius			
ATCC13419	40 (38)	17 (45)	48 (85)
S. sanguis			
804	17 (41)	62 (84)	70 (100)
ATCC10558	11 (25)	53 (98)	64 (100)
NSW13	17 (21)	39 (73)	67 (95)
NSW44	11 (22)	54 (86)	66 (100)
NSW57	12 (33)	43 (73)	66 (100)
L. mesenteroides			
B-512(F)	0	94	94
B-1355(S)	0		

[a]Values for solubilization are given in parentheses.

A comparison of the limit of hydrolysis of each glucan to D-glucose by *C. resinae* (1→3)-α-D-glucanase (Table II) with the content of (1→3)-linkages determined by methylation analysis (Table I), shows that a high proportion of the (1→3)-D-glucosidic linkages in each glucan is susceptible to hydrolysis. As the enzyme specifically recognizes sequences of (1→3)-linked α-D-glucose residues[9], and is unable to hydrolyse (1→3)-linkages that are flanked by (1→6)-α-D-glucosidic linkages in mixed-linkage polysaccharides[9], the enzymic analysis always gives lower values for (1→3)-linkages than does methylation analysis, because of the influence of adjacent linkages. The agreement between the two methods of analysis is sufficiently close to indicate that the majority of the (1→3)-linkages in *S. mutans* α-D-glucans are arranged in sequences. The value for the single strain of *S. salivarius* is within the range obtained with *S. mutans*, whereas the *S. sanguis* strains produce glucans containing a far lower proportion of (1→3)-linked sequences, as shown by the limit of enzymic hydrolysis (range 11–17%). Methylation analysis, unless it is supported by enzymic or other fragmentation studies, cannot give information on the arrangement of the linkages in polysaccharides, and accordingly there is no agreement between the methylation and

enzymic analysis of *L. mesenteroides* B-1355 soluble dextran, where none of the (1→3)-α-D-glucosidic linkages occurs in sequence[31].

Analysis of (1→6)-α-D-glucosidic linkages. — Methylation analysis (Table I) gives values for 2,3,4-tri-*O*-methylglucose which indicate that the content of (1→6)-D-glucosidic linkages in *S. salivarius* and *S. mutans* glucans (range 11–39%) is generally small compared with that of (1→3) linkages. By contrast, (1→6)-D-glucosidic linkages preponderate in *S. sanguis* glucan. As the majority of the (1→3)-linkages in all of the glucans are consecutive, the (1→6)-linked glucose residues are also likely to occur in sequences. Such sequences would be susceptible to hydrolysis by dextranases provided that the proportion of branch linkages is not excessive. A bacterial endodextranase (dextranase-CB) was selected to test for sequences, because this (1→6)-α-D-glucanase

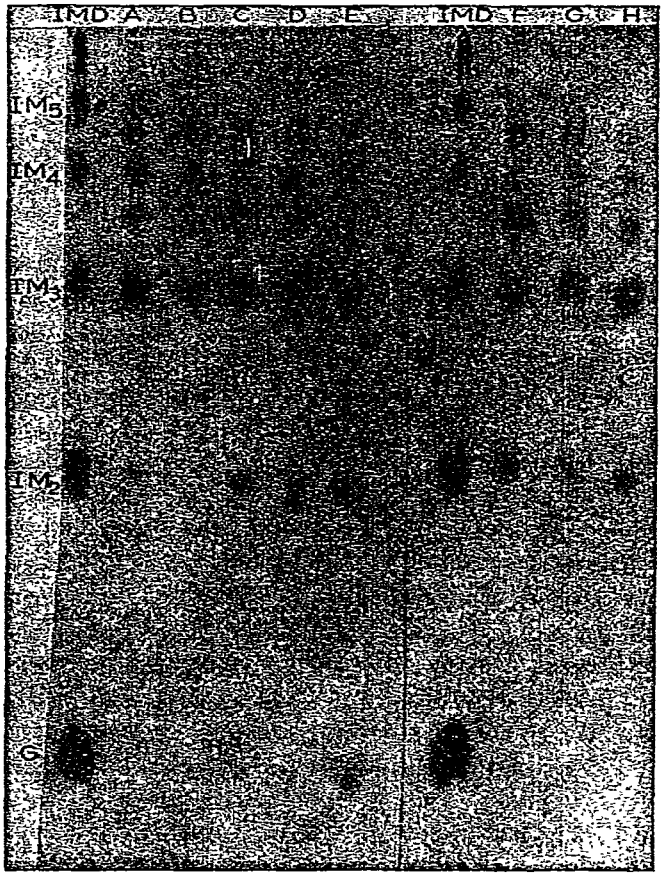

Fig. 1. Separation by paper chromatography of the products of dextranase-CB action on α-D-glucans: *S. mutans* JC2 soluble (A); *S. mutans* JC2 insoluble (B); *S. mutans* NSW1 (C); *S. mutans* NSW47 insoluble (D); *S. mutans* Ingbritt (E); *S. sanguis* NSW13 (F); *S. sanguis* NSW44 (G); and *S. sanguis* 804 insoluble (H). The standard (IMD) contains oligosaccharides of the isomaltose series: G, glucose; IM_2, isomaltose; IM_3, isomaltotriose, and so on.

has no action on (1→3)-α-D-glucan, and the active region of the enzyme contains nine subsites[32]. Thus, a sequence of nine glucose residues is required to occupy the active region fully, and isomaltohexaose is the smallest substrate that can be rapidly hydrolysed[23]. The major products from (1→6)-α-D-glucan are IM_3, IM_4, and IM_5; IM_2 arises from the slow hydrolysis of IM_5. The limit of hydrolysis of several glucans by dextranase-CB is shown in Table II. The values for *S. salivarius* and *S. mutans* glucans are low compared with those for *S. sanguis*, and in some strains (such as B13, K1-R) the limit of enzymic hydrolysis gives little indication of the content of (1→6)-linkages known to be present from methylation analysis. Examination of paper chromatograms showed that only traces of products of low mol. wt. were released from glucans of *S. salivarius* or *S. mutans* OMZ176, and no products at all were seen from *S. mutans* K1-R or B13 glucans. All other glucans, whether they originated from *S. mutans* or from *S. sanguis*, gave identical patterns (Fig. 1) and the major products were oligosaccharides having mobilities corresponding to IM_2, IM_3, and IM_4, together with tetra- and pentasaccharides having mobilities intermediate between IM_3 and IM_4 (B4), and between IM_4 and IM_5 (B_5), respectively. The B series refers to oligosaccharides containing a (1→3) linkage which gives an increased R_F value over an isomaltosaccharide of equivalent d.p.

The appearance of mixed-linkage oligosaccharides together with the usual products (IM_3 and IM_4) of dextranase-CB action[23] on (1→6)-α-D-glucan indicated that the (1→6)-linked chains of the glucans might be branched. Indeed, when *S. sanguis* 804 glucan is considered, the addition of the content of branch points (15% of 2,4-di-*O*-methylglucose) to the content of (1→6)-linked glucose residues (49% of 2,3,4-tri-*O*-methylglucose), gives a value that is close to the limit of hydrolysis by dextranase (62%). This comparison suggests that most of the branching occurs in the (1→6)-linked glucan chains. The values for 2,3,4-tri- and 2,4-di-*O*-methylglucose (Table I) obtained from methylation and hydrolysis of *S. salivarius* and several *S. mutans* glucans are equivalent, indicating that almost half of the D-glucose residues of the (1→6)-linked sequences could bear side chains. This would explain the resistance of these glucans to hydrolysis by dextranase-CB.

Methylation analysis of the soluble glucan synthesized by GTF-S isolated from culture filtrates of *S. mutans* OMZ176 is also consistent with a (1→6)-linked α-D-glucan backbone, with half of the glucose residues bearing glucosyl side-chains attached by a (1→3)-branch linkage. Partial acid hydrolysis of the soluble glucan (0.24M sulphuric acid for 90 min at 100°) yielded glucose, traces of isomaltose and isomaltotriose, and stronger spots corresponding to isomaltotetraose and higher isomaltosaccharides having d.p. up to eleven, as observed on paper chromatograms (higher products were not resolved). This distribution of products supports the concept of a (1→6)-linked main glucan chain. Several other structures that are also consistent with the methylation analysis must be discarded, either because they allow sequences of only two or three (1→6)-linked glucose residues, or because they would be susceptible to hydrolysis by exodextranases (see later).

Enzymic degradations with other endodextranases that are also free from

(1→3)-α-D-glucanase activity, provided results in support of those obtained with dextranase-CB (Table III). The (1→6)-endoglucanases all gave similar values for the limit of hydrolysis of *S. sanguis* 804 glucan, but *S. mutans* dextranases were less active towards some *S. mutans* glucans than dextranase-CB, indicating the greater ability of the latter enzyme to hydrolyse (1→6) linkages that are in the vicinity of branch points. None of the glucans was susceptible to hydrolysis by the exodextranase of *S. mitis*. This enzyme is highly specific for (1→6)-linkages, and its action stops one D-glucose residue away from a branch point[33]. Another exodextranase, the isomaltohydrolase of *A. globiformis*, was slightly more active than the glucohydrolase, because its broader specificity allows the by-passing or hydrolysis of certain branch linkages[34]. Nevertheless, the higher resistance of all of the glucans to the exodextranases compared to endodextranases (Table III), is indicative of branched (1→6)-glucan chains.

TABLE III
ACTION OF BACTERIAL ENDO- AND EXO-DEXTRANASES ON α-D-GLUCANS

Source of glucan	*Limit of hydrolysis* (%)			
	Endodextranases		*Exodextranases*	
	S. mutans *K1-R*	S. mutans *OMZ176*	S. mitis *439*	A. globiformis *T6*
Synthetic dextran	99	100	35	68
Leuconostoc spp.				
B-512(F)	100		23	
B-1355(S)			0	58
B-1355(L)	84	90		34
Dextran 2000	100	100	23	45
Streptococcus spp.				
OMZ176		10	1.0	1.4
Ingbritt		19	2.0	2.8
ATCC13419		5	1.7	9.0
804	72	66	1.4	7.3
Glucosyltransferases				
OMZ176 GTF-S	17		1.0	0
OMZ176 GTF-S+I		7	0	0
K1-R GTF-I	1			

Solubilization of α-D-glucans with endoglucanases. — Incubation of glucans with (1→3)-α-D-glucanase of *C. resinae* generally resulted in more extensive solubilization than hydrolysis. The conversion of the (1→3)-α-D-glucan chains into D-glucose apparently released a proportion of the potentially soluble (1→6)-glucan chains. The action of dextranase-CB on the glucans also gave higher solubilization than expected from the apparent conversion into isomaltotriose. This was partly due to the release

of oligosaccharides of d.p. higher than IM_3, which were thus underestimated. Hydrolysis of the (1→6)-glucan sequences might also have released some (1→3)-linked chains that were sufficiently short, in some instances, to be soluble.

The extent of solubilization of the glucans by dextranase-CB was highly dependent on the strain, ranging from 21 to 98%, and this explains why the success of dextranase treatment in reducing caries in infected animals depends so much on the nature of the infecting strain of *Streptococcus*. In most cases, the combined action of two glucanases was necessary to effect complete solubilization of the glucans (Table II) Separation of the solubilized products by paper chromatography (Fig. 2) revealed all the oligosaccharides released by dextranase alone (Fig. 1), together with a large spot of glucose, and a small spot corresponding to nigerose. As the ability to synthesize insoluble glucan is an important factor in the colonization of *S. mutans*[11], the fact that only two enzymes are required to solubilize water-insoluble glucans from a variety of streptococci has practical significance. Newbrun and Sharma[35] have recently reported that *P. funiculosum* dextranase and *Trichoderma harzianum* mutanase solubilize a series of *S. mutans* glucans (range 62–93%). In large-scale trials of the anti-caries effect of administering glucanases, the use of the constitutive (1→3)-α-D-glucanase of *C. resinae* would be an advantage, because the organism releases enzyme in abundance with no need for a (1→3)-α-D-glucan inducer[36].

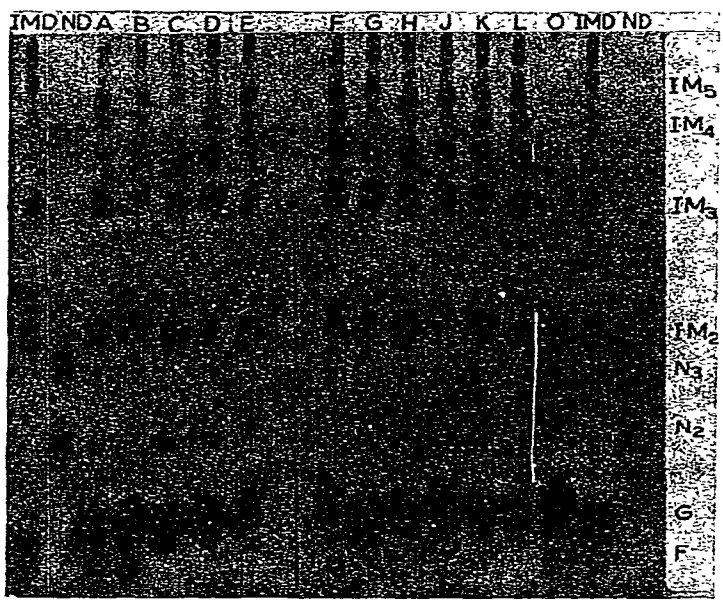

Fig. 2. Separation by paper chromatography of the products of the simultaneous action of *C. resinae* endo-(1→3)-α-D-glucanase and dextranase-CB on α-D-glucans: *S. mutans* JC2 soluble (A); *S. mutans* JC2 insoluble (B); *S. mutans* Ingbritt (C); *S. mutans* NSW1 (D); *S. mutans* NSW47 (E); *S. sanguis* 804 insoluble (F); *S. sanguis* ATCC10558 (G); *S. sanguis* NSW10 (H); *S. sanguis* NSW13 (J); *S. sanguis* NSW44 (K); *S. sanguis* NSW57 (L); and *S. salivarius* ATCC13419 (O). The standards (IMD and ND) contain oligosaccharides of the isomaltose and nigerose series.

Enzymic synthesis of branched (1→6)-α-D-glucan. — Final proof of the structure of the extracellular α-D-glucans of oral strains of *Streptococcus* spp. can only be achieved by their synthesis. To this end, it is necessary to study the properties and action patterns of isolated D-glucosyltransferases that contain only one activity, as shown by linkage analysis of the product of their reaction with sucrose. The interaction between the individual enzymes during the synthesis of insoluble glucan can then be investigated, so that the mechanisms involved in the synthesis of a product having the same structural characteristics as native glucan may be understood.

The co-existence in *Streptococcus* spp. glucans of sequences of (1→3)-linked glucose residues and branched (1→6)-α-D-glucan chains, as demonstrated by degradative methods, is consistent with the separation from cell-free filtrates of many strains of *S. mutans* of two fractions[22], which catalyze the synthesis of soluble and insoluble glucan, respectively. A dextransucrase isolated[37] from *S. mutans* HS-6 produced a soluble glucan containing 94% of (1→6)-α-D-glucosidic linkages, as determined by its consumption of periodate, and the glucan prepared with *S. mutans* GS5 dextransucrase was also reported to be predominantly (1→6)-linked[38]. A dextransucrase[39] from *S. mutans* 6715, which synthesizes a soluble glucan[40], bears a resemblance to GTF-S from *S. mutans* OMZ176, and the two enzymes have been isolated by similar techniques.

An important feature of the activity of the dextransucrase of *S. mutans* 6715 is its dependence on dextran. Synthesis of soluble glucan in the absence of added dextran occurred only after an extended lag-period, whereas the addition of $2–3 \times 10^{-6}$M dextran gave half-maximal stimulation of dextran synthesis[41]. D-Glucosyltransferase-S from *S. mutans* OMZ176 displayed a similar lag (Fig. 3) and this was totally abolished by small quantities of dextran 2000, with 50% of maximal stimulation occurring in 1×10^{-8}M dextran. Synthesis in the absence of dextran began after 8 h, and continued until all the sucrose was converted into soluble glucan (80 h). No

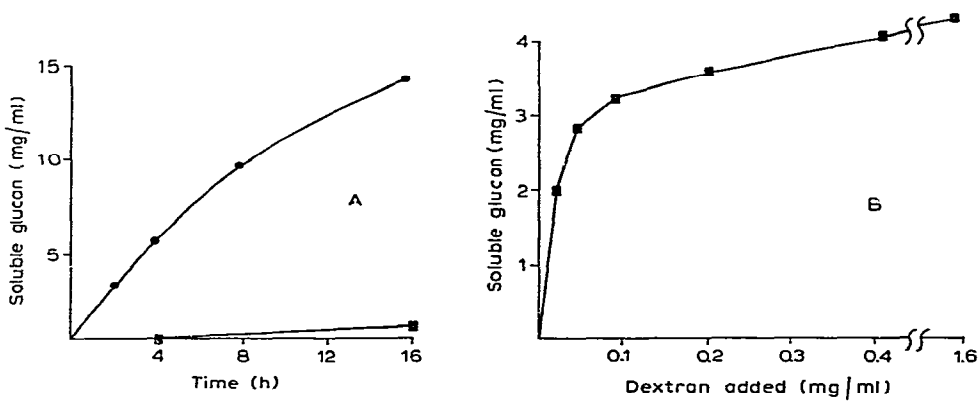

Fig. 3. Effect of dextran on the synthesis of soluble glucan by *S. mutans* GTF-S. A, With sucrose alone (■), and with 0.4 mg of dextran 2000 per ml (●), B, Incubation for 3 h with sucrose, together with increasing concentration of dextran 2000.

activation occurred when dextran was replaced by (1→3)-α-D-glucan or by water-insoluble glucan (mutan) of *S. mutans* OMZ176. By contrast, soluble glucan of *S. mutans* (synthesized by GTF-S) was 50% as efficient as dextran 2000 in the stimulation of synthesis. The enzyme activity is therefore not accelerated by insoluble glucans.

Enzymic synthesis of (1→3)-α-D-glucan. — Synthesis of (1→3)-α-D-glucan proceeded without lag when D-glucosyltransferase-I (0.03 unit) was incubated with sucrose in the absence of added polysaccharide. Addition of small amounts of (1→3)-α-D-glucan resulted in variable degrees of inhibition, whereas the rate of the reaction was markedly increased in the presence of dextran (Fig. 4). This result thus provides an example of a polymerase that is stimulated by a glucan containing a linkage different from that in the product synthesized by the enzyme. Another consequence of the presence of (1→6)-glucan became apparent when an excess of dextran was added. At dextran concentrations up to 0.4 mg/ml, the product of GTF-I activity was all water-insoluble; thereafter the proportion of water-insoluble glucan fell sharply. It may be inferred that the product of GTF-I activity was now soluble because the proportion of (1→3)-linked D-glucose sequences synthesized by the enzyme was outweighed by that of the (1→6)-linked sequences supplied by dextran. The activating effect of dextran is thus multi-functional, because the ability of dextran to keep the growing chains of (1→3)-glucan in solution permits GTF-I to remain in the soluble, and hence, active state. The adsorption[10,42] and irreversible inactivation[43] of the D-glucosyltransferases of *S. mutans* onto water-insoluble glucan has been well documented. The solubilizing effect of dextran on potentially insoluble glucan has previously been observed in similar experiments with preparations from *S. mutans*

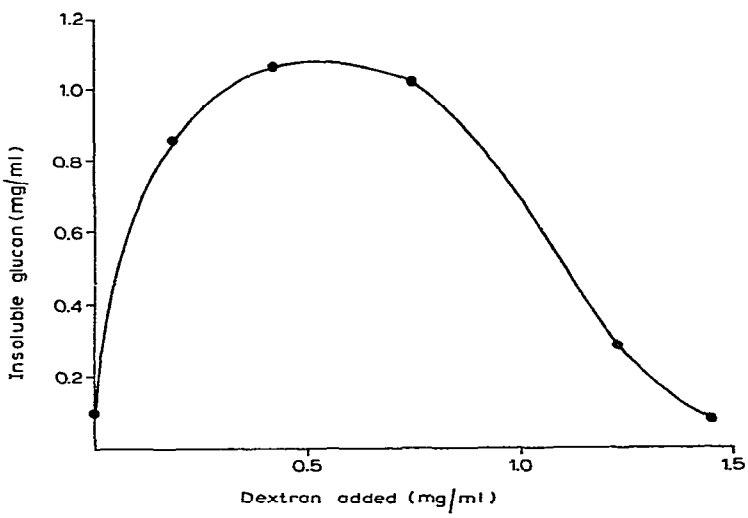

Fig. 4. Effect of dextran (mol. wt. 2×10^6) on the synthesis of water-insoluble glucan by *S. mutans* OMZ176 GTF-I. The enzyme (0.03 unit) was incubated with sucrose (10 mg) for 30 min at 35°.

K1-R (ref. 43) and OMZ176 (ref. 44) that most likely contained both GTF-S and GTF-I activity. Robyt and Corrigan[44] considered the phenomenon in terms of an increased rate of nucleophilic displacement of dextranosyl chains with increasing concentration of exogenous dextran. They claimed that this would allow the transfer of short, soluble chains and inhibit the synthesis of long, insoluble, dextran chains. The probability that their dextransucrase preparation contained GTF-I activity was not reported.

The fact that the synthesis of (1→3)-α-D glucan chains can give a water-insoluble product when such chains are minor features of a predominantly (1→6)-linked α-D-glucan (as in *S. sanguis* glucans), explains why both soluble and water-insoluble glucans can be produced when culture filtrates of *S. mutans* are incubated with sucrose. The insoluble glucan of *S. mutans* NSW47 and *S. sanguis* 804 containep more (1→3) linkages and fewer (1→6) linkages than the soluble glucans (Table I). Methylation analysis[7] of *S. mutans* JC2 soluble glucan revealed the presence of 33% of (1→3)-α-D-glucosidic linkages not involved in branching. Moreover, the (1→3)-α-D-glucanase of *C. resinae* released substantial amounts of glucose from both the soluble and insoluble glucans of *S. mutans* JC2 (Fig. 2A, B). These soluble glucans are thus totally different from the glucans synthesized by GTF-S, which do *not* contain sequences of (1→3)-linked D-glucose residues (Tables I and II). It follows that a determination of the weight of insoluble and soluble glucan synthesized from sucrose does not accurately reflect the activity of GTF-I and GTF-S, respectively, in unfractionated, cell-free filtrate.

A comparison of the efficiency of equal weights (50 μg) of various dextrans in the stimulation of GTF-I activity (Table IV) has provided insight into some of the factors involved in the activation. The amount of water-insoluble glucan synthesized by GTF-I isolated from *S. mutans* K1-R increased slightly with the mol. wt. of acid-

TABLE IV

EFFECT OF ADDED POLYSACCHARIDES ON GLUCOSYLTRANSFERASE-I ACTIVITY

Additions	*Insoluble glucan (counts/min)*	*Activation*
None	609	
Inulin	648	1
S. salivarius fructan	636	1
B-1355(S) dextran	1055	1.7
OMZ176(S) glucan[a]	9692	16
B-512 dextran (acid-degraded)		
mol. wt. 1.8×10^4	12143	20
2.3×10^5	19100	31
2.0×10^6	17805	29
Synthetic (1→6)-α-D-glucan		
mol. wt. 3.2×10^4	14022	23

[a]Synthesized with GTF-S.

hydrolyzed fractions of *L. mesenteroides* B512(F) dextran. A chemically synthesized (1→6)-α-D-glucan[28] (d.p. 150), that is virtually free from anomalous linkages, stimulated the reaction 23-fold, whereas a B512(F)-dextran fraction (d.p. 100) stimulated the reaction by 20-fold. The presence of side-chains attached by (1→3)-α-D-glucosidic branch-linkages were therefore not inhibitory. By contrast, the activation by a native, soluble dextran (fraction S) produced by *L. mesenteroides* B-1355 was practically negligible, indicating that sequences of (1→6)-linked D-glucose residues may be necessary for the stimulation of GTF-I activity. Such sequences are not a predominant feature of B-1355(S) dextran, which contains 34% of (1→3)-α-D-glucosidic linkages, arranged alternately with (1→6) linkages[2]. The soluble glucan synthesized with GTF-S, which is highly branched, activated synthesis of water-insoluble polysaccharide by 16-fold. This result indicated that GTF-S synthesized a glucan that contained suitable locations where glucosyl residues transferred by GTF-I could be accepted.

The simultaneous action of two D-*glucosyltransferases* (*GTF-S and GTF-I*) *on sucrose.* — The weight of insoluble glucan synthesized by GTF-I was greatly increased by the addition of increasing concentrations of GTF-S (Fig. 5). The activity of GTF-I (0.4 unit) was stimulated by 100% in the presence of only 0.01 unit of GTF-S, and on raising the concentration of GTF-S up to 0.04 unit the synergic effect resulted in a 10-fold increase in the synthesis of insoluble glucan. Thus, quite small amounts of either GTF-S or dextran were extremely effective activators for GTF-I. In a separate experiment, in which the effect of a 25-fold excess of GTF-S (75 milli-units) on the activity of GTF-I (3 milli-units) was investigated, the presence of GTF-S raised the

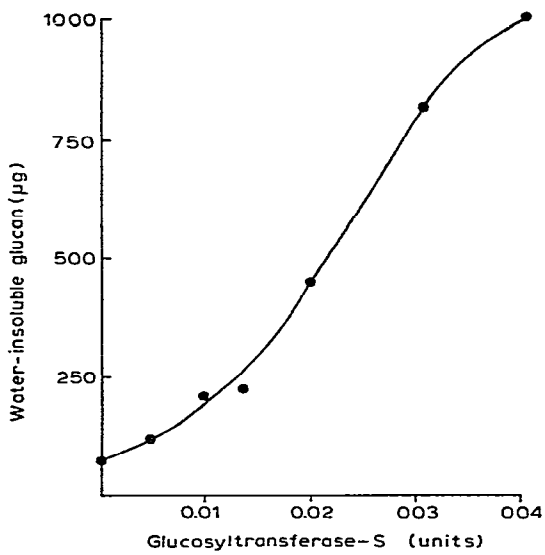

Fig. 5. Weight of water-insoluble glucan synthesized from sucrose by *S. mutans* OMZ176 GTF-I (0.4 unit) in the presence of increasing concentrations of GTF-S.

production of insoluble glucan from 68 μg (GTF-I alone) to 1436 μg. At the same time, the weight of soluble glucan fell from 1798 μg (with GTF-S alone) to 847 μg. Experiments in which various proportions of both GTF-I and GTF-S were incubated with sucrose showed that the amount of GTF-I required to synthesize 1 mg of water-insoluble glucan could be decreased from 1 unit to 0.25 unit in the presence of 0.1 unit of GTF-S.

It is probable that several factors are involved in the synergic effectsobserved when GTF-I reacted with sucrose in the presence of GTF-S, and that two of these were similar to those already noted with dextran. The presence of GTF-S ensured a *constant* supply of new chains of (1→6)-α-D-glucan, which not only stimulated GTF-I activity, but also promoted the solubility of the product, thereby postponing the end of the synthetic reactions, which would occur when the glucosyltransferases were irreversibly inactivated by adsorption onto insoluble glucan.

Biosynthesis of α-D-*glucans of* Streptococcus *spp.* — The insertion mechanism of dextran biosynthesis proposed by Robyt *et al.*[44,46] for *L. mesenteroides* B-512(F) dextransucrase accounts for the synthesis of (1→6)-α-D-glucosidic linkages and the (1→3)-α-D-glucosidic branch linkages of B-512(F) dextran. The same D-glucosyltransferase is involved with both functions. Germaine *et al.*[41], who separated dextransucrase (GTF-S) from *S. mutans* 6715 into two fractions by gel electrophoresis, considered that the dextran-dependent enzyme activity in the minor fraction represented a branching enzyme that transferred D-glucose residues from sucrose to (1→6)-α-D-glucan, with the formation of a (1→3)-α-D-glucosidic branch-linkage. If this suggestion were verified by experimental evidence, then the variation in branch-linkage content of soluble glucans synthesized by GTF-S preparations from different serotypes of *S. mutans* could be explained by differences in the branching-enzyme activity of the strains. This factor could be one of many responsible for the variable effects of dextranase on hydrolysis of D-glucan *in vitro* (Table II), and on the dispersion of plaque[45] and reduction of caries *in vivo*. However, as linear and branched (1→6)-α-D-glucans both markedly stimulated GTF-I activity (Table IV), the synthesis of water-insoluble glucan following the interactions between GTF-S and GTF-I most probably constitutes a mechanism that is operative for all serotypes of *S. mutans*.

The question as to how the (1→6)-linked and (1→3)-linked sequences are attached to one another is of considerable interest. By analogy with the acceptor reaction involved in the introduction of side-chains into B-512(F) dextran[46], the possibility that attachment occurs by branching deserves consideration. McCabe and Smith[47] concluded that extracellular enzymes of *S. mutans* K1-R modified (1→6)-α-D-glucan chains to become more-efficient acceptors for dextransucrase through the introduction of additional branch linkages. It is already known that a preparation of GTF-I from *S. mutans* OMZ176 contains an activity that catalyzes the transfer of D-glucosyl residues from sucrose to isomaltosaccharides[48]. The branched oligosaccharides that are produced contain a 1-unit side-chain attached by a (1→3)-α-D-glucosidic linkage to the glucose residue penultimate to the reducing end[32]. It is also possible that the linear (1→3)-α-D-glucan chains synthesized by GTF-I are transferred

to (1→6)-α-D-glucan chains by a displacement reaction in which a C-3 hydroxyl group of the dextran acceptor makes a nucleophilic attack on C-1 at the reducing end of a (1→3)-α-D-glucan chain, thus forming a (1→3)-α-D-glucosidic branch linkage. If a similar transfer of (1→6)-α-D-glucan chains onto an acceptor consisting of (1→3)-linked glucose sequences could also occur, giving structures similar to that described by Ebisu *et al.*[5], then repeated transfers of these two kinds would result in the formation of a highly branched, mixed-linkage glucan. Such a hypothesis accounts for the synergy observed between GTF-S and GTF-I, and explains why dextrans modified at either the reducing[49] or the nonreducing ends[44] continue to activate the reaction. The availability of hydroxyl groups on a *sequence* of inner glucose residues provides a multivalent acceptor for the transferred chains.

Structure of α-D-glucans. — The simplest possible average repeating-unit for glucans of *S. mutans* would contain one (1→3)-linked chain and one (1→6)-linked chain. This is represented diagrammatically in Fig. 6A, where each chain is attached to another by means of a branch linkage. The structure is drawn to account for the results of methylation analysis of *S. mutans* OMZ176 glucan, but it is clearly not consistent with the susceptibility of the glucan to hydrolysis by dextranase-CB. No known endodextranase could attack the (1→6)-α-D-glucosidic linkages depicted in

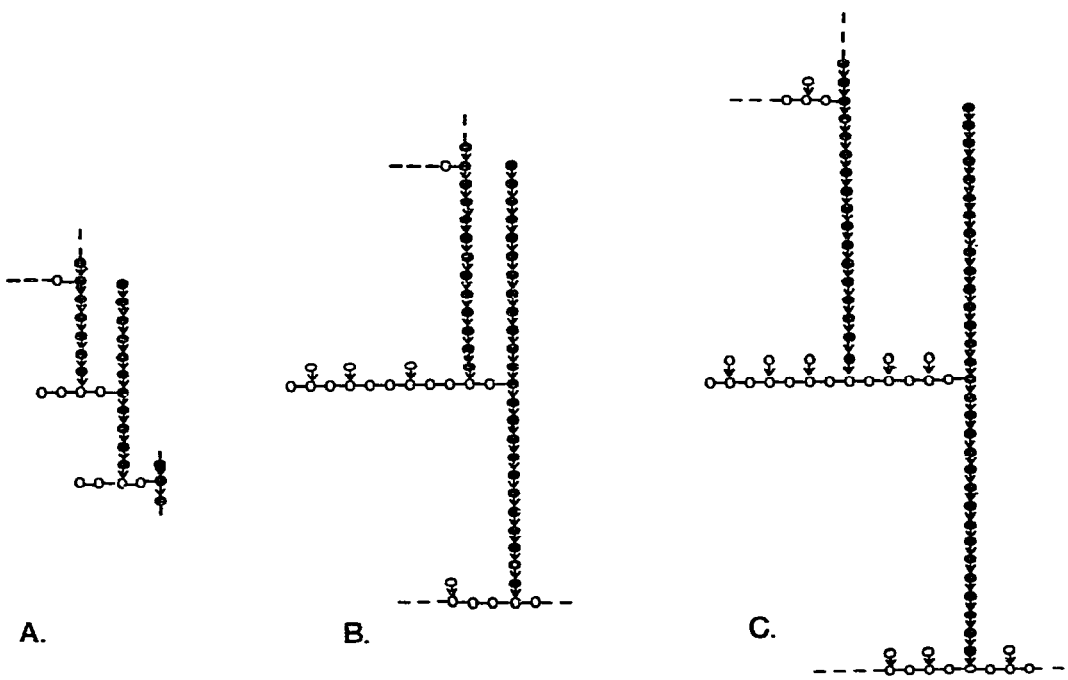

Fig. 6. Symbolic representations of fragments or average repeating-units proposed for *S. mutans* OMZ176 water-insoluble glucan, showing equal numbers of two types of chain. ○, D-glucose residue from a branched (1→6)-α-glucan chain; ———, (1→6)-α-D-glucosidic linkage; ●, D-glucose residue from a (1→3)-α-glucan chain; and ↓, (1→3)-α-D-glucosidic linkage.

Fig. 6A. An extension of the length of the (1→6)-linked chain is obviously required, and this demands a corresponding increase in the number of branch linkages and in the length of the (1→3)-linked sequences (Fig. 6, B and C). The additional branch points have been added to the (1→6)-α-D-glucan chains, in keeping with the synthesis of branched chains by GTF-S, and with the synthesis of a relatively linear (1→3)-α-D-glucan by GTF-I. When the (1→3)-glucan chains are attached close to the reducing end of the branched (1→6)-glucan chains, as in Fig. 6B, the structure resembles that proposed by Ebisu *et al*[5], in which short (1→6)-linked side-chains are attached to the main chain of (1→3)-α-D-glucan. However, it is more likely that the glucan would consist of a variety of structures, including all those shown in Fig. 6, and that the position where (1→6)-D-glucan chains are attached to (1→3)-D-glucan chains would also vary. Furthermore, many of the chains might bear multiple side-chains, giving a highly ramified structure. The proposal has the merit that the same general structure, depicted in Fig. 7C, can be applied to both *S. mutans* and to *S. sanguis*

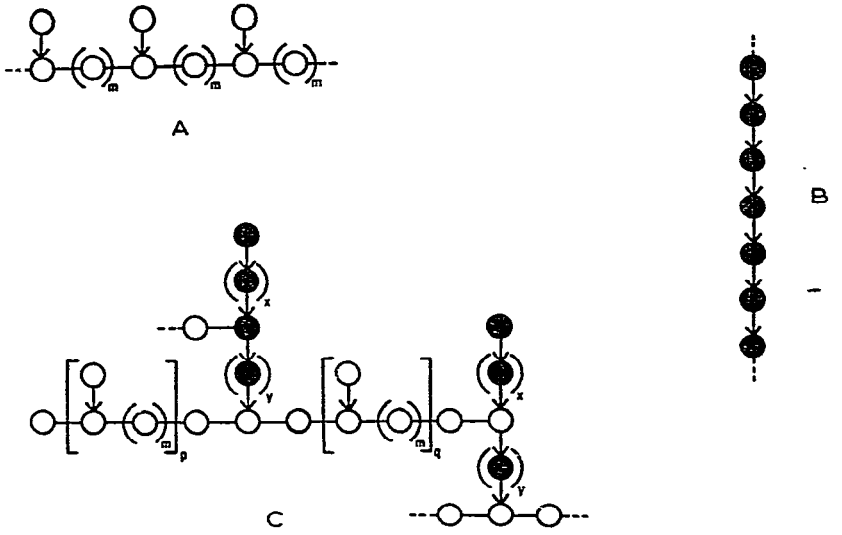

Fig. 7. A, General formula proposed for soluble glucan synthesized by GTF-S. The average value of *m* is close to 1 for *S. mutans* OMZ176 glucan. B, Sequence of (1→3)-linked D-glucose residues synthesized by GTF-I. C, General structure proposed for glucan synthesized from sucrose by the combined action of GTF-S and GTF-I. The relative values of *m*, *p* and *q*, *x* and *y* are strain-dependent. The symbols are the same as in Fig. 6.

glucans. It accounts for the diversity that exists between glucans from different strains of *S. mutans*, whereas *S. sanguis* glucans, with their excess of (1→6)-linkages, take their place at the end of the range, with high values of *m* (~3–5), and low values for $(x+y)$. At the other extreme, some *S. mutans* strains (such as K1-R) produce glucans in which $(x+y)$ greatly exceeds $(p+q)$, and the value of *m* may fall to 1, giving glucans that are totally resistant to dextranase-CB. Although the (1→6)-linked glucan

chains of *S. mutans* are short, they are generally highly branched, so that analysis of *S. mutans* glucans reveals a similar degree of branching to that of *S. sanguis* glucans, in which the (1→6) linked glucan chains, which bear fewer branches, are predominant. Other, detailed, chemical analyses[50] have failed to show any essential difference between the soluble glucans elaborated by cariogenic and less-cariogenic streptococci.

A structural analysis of insoluble glucans produced by several cariogenic strains of *S. mutans*, including OMZ176, was based on methylation analysis and periodate oxidation[51]. It was concluded that the glucans contained two types of polysaccharide chain, a (1→3)-α-D-glucan and a dextran-like polysaccharide having single, (1→3)-linked D-glucose side-chains. The results of another study of *S. mutans* OMZ176 glucan, based similarly on methylation analysis and the Smith degradation, indicated that most of the (1→6)-linkages occurred as side-chains that contained 1–3 glucose residues and which were attached to the main (1→3)-glucan chain to form a comb like structure[5]. These short, (1→6)-linked side-chains would be resistant to hydrolysis by endodextranases, but more susceptible to exodextranases. Experimentally, we have shown the reverse to be true. In addition, the main (1→3)-linked glucan chain, being branched at frequent intervals (a side-chain is attached to every fourth or fifth D-glucose residue,) would be totally resistant to exo-(1→3)-glucanase, and the hydrolysis by *C. resinae* (1→3)-endoglucanase would also be severely restricted. In practice, we found that the (1→3)-α-D-glucanase of *C. resinae* could hydrolyze the majority of the (1→3)-linked sequences, without release of the isomaltosaccharides that would result from hydrolysis of (1→3) linkages adjacent to the branch points. Furthermore, the (1→3)-exoglucanase of *Aspergillus nidulans* has been reported[19] to release glucose from the glucan of *S. mutans* GS5. The present study has thus emphasized the unique role of enzymic analyses in exploring the structure of the glucans. The structure depicted by Fig. 7C is consistent with the results of hydrolyses with glucanases of known specificity, and accounts for the similarity of the products released from the glucans of *S. mutans* and *S. sanguis* strains. Moreover, the structure is compatible with the observed stimulation of GTF-I activity by (1→6)-α-D-glucans and by GTF-S, whereas these phenomena are not easily interpreted in terms of a comb-like structure.

The D-glucosyltransferases of *S. sanguis* have not been separated into two fractions capable of synthesizing (1→6)-linked glucan (as in Fig. 7A) and (1→3)-linked glucan (as in Fig. 7B), respectively, but there are strong indications that two such activities are produced[6,52]. Two reasons can be advanced to explain why (1→6)-linked chains are predominant in *S. sanguis* glucans. Firstly, *S. sanguis* strains do not produce dextranase[26]. Secondly, a GTF-S type activity may be produced in great excess over GTF-I. Even if *S. sanguis* and *S. mutans* were to produce equivalent proportions of GTF-S and GTF-I, the endogenous dextranases of *S. mutans*, by trimming down the (1→6)-glucan chains, could account for the predominance of (1→3)-linked sequences in *S. mutans* glucans. It is noteworthy that α-D-glucans from *S. mutans* strains having the highest dextranase activity (K1-R, B13) produce glucans with the highest proportion of (1→3)-linked sequences and which are the least

susceptible to further degradation by dextranase-CB (Table II). On the other hand, dextranase-defective mutants of *S. mutans* apparently share with *S. sanguis* the property of being less-cariogenic than wild-type cells of *S. mutans*[53]. Other mutants of *S. mutans* that exhibit decreased synthesis of water-insoluble glucan but continue to produce soluble glucan, are also less cariogenic[16–18], and produce plaques that allow free diffusion of acid[54]. It is logical to conclude that the higher cariogenic potential of *S. mutans* relative to *S. sanguis* is related to its capacity to synthesize glucan containing a higher proportion of (1→3)-linked sequences. Long sequences of (1→3) linkages permit the α-D-glucan to adopt a stiff, extended, ribbon-like shape which tends to form fibrous aggregates[55]. This structural feature would effectively contribute to the formation of a plaque that constituted a barrier to the diffusion of acidic fermentation-products away from the tooth surface.

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