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# Enzymic and methylation analysis of dextrans and (1→3)-α-D-glucans

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## ABSTRACT

Chemically synthesized (1→3)-α-D-glucans, dextrans from *Leuconostoc spp.*, and glucans from *Streptococcus sobrinus* strains were examined by enzymic degradation, methylation analysis, and n.m.r. spectroscopy. (1→3)-α-D-Glucans were hydrolyzed completely to D-glucose and nigerose by an endo-(1→3)-α-D-glucanase from *Cladosporium resinae*. The extent of hydrolysis of native *Leuconostoc* dextrans, in which by definition 50% or more of the linkages are (1→6), and of *S. sobrinus* mixed-linkage glucans, was limited, not only by their linkage compositions, but also by the arrangement of the (1→3) and (1→6) linkages and by accessibility to the enzyme. Estimated enzymic conversions of several water-insoluble glucans, which contained 11–63% of (1→3) linkages, agreed with the experimental values when calculated on the basis of a 4% content of branch points involved in the attachment of (1→6)-linked chains to the (1→3)-linked chains, and four resistant linkages for each such interchain branch-point. By contrast, the more-soluble *Leuconostoc* dextrans contained fewer (1→3) linkages (1–5%) and their complete susceptibility to enzymic hydrolysis indicated their peripheral location. Three soluble dextrans, synthesized by three different α-D-glucosyltransferases (GTF-S), isolated from culture filtrates of *S. sobrinus*, contained only small proportions of (1→3) linkages not involved in branching, and they were resistant to the endo-glucanase. The relationship for 37 glucans between their enzymic hydrolysis and the proportion of (1→3) linkages permits reliable and sensitive estimates of the sequences of (1→3) linkages.

## INTRODUCTION

Most dextrans are synthesized from sucrose by the Lactobacillaceae, notably by certain species of the genera *Leuconostoc*[1] and *Streptococcus*. Dextrans are α-D-glucans in which more than 50% of the linkages are (1→6). Certain water-insoluble glucans of *L. mesenteroides*[2] and the cariogenic *S. sobrinus*[3–5] also contain sequences of (1→3)-linked α-D-glucosyl residues, and a glucosyltransferase (GTF-I) that catalyzes the synthesis of (1→3)-α-D-glucans has been isolated from several strains of *S. sobrinus*[4,6]. A combination of methylation analysis and enzymic degradation with endo-(1→3)-α-D-

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glucanase[7] has been used to examine the distribution of (1→3) linkages in water-insoluble α-D-glucans synthesized by oral streptococci grown in batch culture[4]. The limit of enzymic hydrolysis agreed well with that predicted from the results of methylation analysis for the (1→3)-α-D-glucans synthesized by GTF-I from *S. sobrinus* strains OMZ176 and K1-R. However, the enzyme failed to cleave all the (1→3) linkages in glucans synthesized by the mixture of enzymes in unfractionated culture filtrates. In these glucans, the (1→3)-linked chains are attached to (1→6)-linked chains that are synthesized by a different type of glucosyltransferase (GTF-S).

Some new glucans have become available recently. Three different types of GTF-S, separated[8] from *S. sobrinus* strains grown in complex media supplemented with Tween 80, catalyzed the synthesis of structurally distinct dextrans (S1, S3, and S4). A specific analysis for (1→3;*l*,*c*)-α-linked residues* in these dextrans has not been reported. New glucans have been prepared from oral streptococci grown in continuous culture under defined conditions[8]. Because the conditions of growth govern the activity and distribution of the glucosyltransferases (GTF-S and GTF-I) released by *S. sobrinus* strains[8], incubation of their culture filtrates with sucrose resulted in the synthesis of water-insoluble α-D-glucans in which the proportions of (1→6) and (1→3) linkages were different for each steady state. Consequently, the percentage of (1→3)-linked residues, whether they be (1→3;*l*) or (1→3;*b*), cannot be constant for these polysaccharides. Methylation analysis has revealed a range of structures for glucans from strains K1-R and OMZ176, grown under various conditions[8]. A simple, convenient method for estimating the (1→3;*l*,*c*)-linked residues synthesized by GFT-I is needed, not only for exploring the structure of *S. sobrinus* glucans, but also for screening insoluble dextrans from other streptococci and *Leuconostoc* spp.

We now report on the analysis of the new glucans. For this purpose, the specificity of the endo-(1→3)-α-D-glucanase from *Cladosporium resinae* was characterized further by determining its activity towards (*a*) chemically synthesized, highly stereoregular D-glucans with 100% of (1→3) linkages; and (*b*) a range of *Leuconostoc* dextrans containing from 1–40% of (1→3)-linked units.

## MATERIALS AND METHODS

*Polysaccharides.* — A (1→3)-α-D-glucan was obtained[9] by polymerization of 1,3-anhydro-2,4,6-tri-*O*-(*p*-bromobenzyl)-β-D-glucopyranose, using trifluoromethanesulphonic anhydride as initiator, followed by removal of the protecting groups. Likewise, a slightly less stereoregular (1→3)-glucan was obtained from 1,3-anhydro-2,4,6-tri-*O*-benzyl-β-D-glucopyranose[9]. Pseudonigeran was isolated from *Aspergillus niger* NRRL 326 as described[7], and a soluble carboxymethyl derivative of *A. niger* (1→3)-α-D-glucan was kindly provided by Dr. H. J. Phaff. Glucan from tramal tissue of *Polyporus betulinus* was a generous gift from Dr. E. T. Reese.

* The linkage designations are given in ref. 5: *l*, linear (*i.e.*, unbranched); *c*, contiguous; *b*, branched; *nc*, not contiguous.

The preparation and properties of dextrans, primarily from the NRRL B-strains of *L. mesenteroides*, have been described[1]. Three types of soluble dextrans from *S. sobrinus* were prepared by incubating sucrose with three different D-glucosyltransferases that were isolated from culture filtrates by chromatography on hydroxyapatite[8]. Water-soluble and water-insoluble mixed-linkage glucan fractions were prepared by incubating sucrose with unfractionated culture filtrates obtained from continuous cultures of *S. sobrinus*[8].

*Preparation of endo-(1→3)-α-D-glucanase (EC 3.2.1.59).* — *C. resinae* QM7998 spores were inoculated into 20 conical flasks (500 mL) each containing a *Trichoderma viride* salts medium[10] (100 mL) supplemented with Tween 80 (0.1%) and soluble starch (0.5%) as carbon source[11]. After 9 days in an incubator-shaker at 29°, the mycelium and cell debris were removed by centrifugation at 34 000*g* for 20 min. The supernatant solution (1964 mL) was then concentrated to 114 mL in a device (Amicon, CH4) fitted with a hollow fibre cartridge (HIP10-8). A portion of the concentrate (52 mL) was dialysed against 20mM sodium phosphate buffer (pH 6.0) and applied to a jacketed column of DEAE-Sephadex (A-50) that had been washed with the same buffer. The column was eluted with a linear gradient of sodium chloride at 10 $mL.h^{-1}$, and the (1→3)-α-D-glucanase activity was eluted at 0.025 — 0.10M chloride. The appropriate fractions were combined, dialysed against 5mM potassium phosphate (pH 6.0), and applied to a column of hydroxyapatite (Bio-Gel HTP) that had been washed with the same buffer. The enzyme was recovered (91%) in the void volume. These two steps were sufficient to remove all activity towards β-(1→3) linkages and α-(1→6) linkages from the enzyme preparation. The separations by liquid chromatography were carried out at 5°. The separation on Bio-Gel HTP replaced the isoelectric focusing step used previously[7], and this reduced the time of preparation by several days. The limits of hydrolysis of water-insoluble glucans by the two preparations were the same.

*Determination of (1→3)-α-D-glucanase activity.* — The enzymic digest (1.0 mL) contained *S. sobrinus* OMZ176 water-insoluble glucan fraction (7 mg), 10mM sodium citrate (pH 4.5), and enzyme. After incubation for 15 and 30 min at 35°, samples were withdrawn into ice-cold centrifuge tubes, and centrifuged for 5 min at 5° at 2000*g*. D-Glucose oxidase[12] reagent (0.75 mL) was added to a portion of the supernatant solution, and the mixture (1.0 mL) was incubated for 1 h at 35°. One unit of enzyme is defined as the amount that liberated 1 μmol of D-glucose per min in the activity digest.

*Endodextranases (EC 3.2.1.11).* — Bacterial endo-(1→6)-α-D-glucanase was isolated[13] from the product supplied by Calbiochem, and *Penicillium funiculosum* dextranase was prepared as described[14]. The dextranase activity of these preparations was determined as described[14] and one unit of enzyme is defined as the amount that liberated 1 μmol of reducing sugar per min under standard conditions.

*Limit of enzymic hydrolysis and solubilization of glucans.* — Each glucan (1 mg) was incubated with enzyme in duplicate digests (0.3 mL) buffered to pH 6.0 for 7 and 9 days at 35° under toluene. Insoluble material was then removed by centrifugation, and the concentration of glucose, reducing sugars, and soluble carbohydrate in the super-

natant solution was determined as described[7]. Hydrolysis by endo-(1→6)-α-D-glucanase (0.022 U) was expressed as the apparent conversion into isomaltotriose and isomaltose for the bacterial and fungal enzyme, respectively, and hydrolysis by (1→3)-α-D-glucanase (0.23 U) was reported as the conversion into glucose. Some soluble products were separated by h.p.l.c. on Dextropak (Waters Associates) as described[8].

*Methylation analysis*[15] *of (1→3) linkages.* — The content of (1→3;*l*)-linked units was given by the mole % of 2,4,6-tri-*O*-methylglucose. The proportion of α-(1→3) linkages in the chemically synthesized glucans was estimated by $^{13}$C-n.m.r. spectroscopy[9], by comparing the areas of the peaks for the resonances of C-1β or C-1α at δ 102.7 and 96.2, respectively.

## RESULTS

*Enzymic hydrolysis of chemically synthesized (1→3)-D-glucans.* — From their method of synthesis[9], it was known with certainty that these glucans were unbranched. The n.m.r. data confirmed that the polymers contained >95% of α linkages. Therefore, such glucans can be used as models for (1→3)-α-D-glucans synthesized by α-D-glucosyltransferases from *S. sobrinus*.

The action of *C. resinae* (1→3)-α-D-glucanase on synthetic glucans was compared with that on the (1→3)-α-D-glucan from *Aspergillus niger* strain 326 (pseudonigeran) and on the mixture of (1→3)-α-D-glucan and (1→3)-β-D-glucan isolated from the tramal tissue of *Polyporus betulinus*. The (1→3)-α-D-glucans became completely soluble in water after the enzymic digestion, but the product from *P. betulinus* was only partly soluble (45%), and the extent of hydrolysis was limited to 36%. The hydrolysis of the soluble, carboxymethyl (CM) derivative of *A. niger* (1→3)-α-D-glucan was limited to 43% conversion into D-glucose (*cf.* 79% for the unsubstituted insoluble glucan). The CM-glucan contained an average of one CM group per six glucose residues and its incomplete hydrolysis was consistent with the two linkages adjacent to the glucose residue bearing the CM group being resistant to enzymic attack.

The products of hydrolysis of the (1→3)-α-D-glucans are shown in Table I. D-Glucose was the major product together with 18–20% of nigerose (3-α-D-glucosyl-D-glucose). The yield of glucose plus nigerose was in good agreement with the percentage of (1→3) linkages expected from n.m.r. analysis. Minor amounts of laminaribiose (3-β-D-glucosyl-D-glucose) were also released from the synthetic glucans, together with oligosaccharides (three peaks in Fig. 1b). The retention times for the oligosaccharides (13.0–13.8 min) were in between those of nigerotriose (10.6 and 12.4 min) and nigerotetraose (22.8 and 25.4 min). Because β-linked disaccharide emerged later (10.1 min) than α-linked disaccharide (7.9 min), it was considered that the 3 oligosaccharide peaks might be αβ-, βα-, and ββ-glucotrioses. Under the conditions of separation, oligosaccharides gave dual peaks due to the α and β anomers[16]; hence, the 3 peaks observed did not necessarily signify three different oligosaccharide products. Their yields were insufficient to allow individual characterization and their identities were checked by incubating glucan DBn11 with endo-(1→3)-α-D-glucanase together with *C. resinae* exo-

(1→3)-α-D-glucanase[17] and exo-(1→3)-β-D-glucanase. Fractionation of the products then revealed a slight increase in glucose, a decrease in nigerose, and loss of laminaribiose and oligosaccharides (Fig. 1c). It was concluded that the best samples of chemically synthesized (1→3)-D-glucans contained >97% of α linkages. The release of laminaribiose indicated the ability of *C. resinae* endo-(1→3)-α-D-glucanase to hydrolyze α linkages that flanked a β residue in these glucans.

TABLE I

Fractionation of the products formed by the action of *Cladosporium resinae* endo-(1→3)-α-D-glucanase on D-glucans

| *Glucan* | *α Linkages* (%) | *H.p.l.c. separations*[a] *(area %)* | | | |
|---|---|---|---|---|---|
| | | *Glc*[b] | *Nigerose* | *Laminaribiose* | *Oligosaccharides* |
| *A. niger* | 100 | 79 | 20 | | 0 |
| DBr 20 | 95–100[c] | 79 | 18 | 0.2 | 2.8 |
| DBr 26 | 95–100[c] | 77 | 19 | 1.2 | 2.8 |
| DBn 11 | 85–90[c] | 66 | 24 | 3.0 | 7.0 |

[a] Eluted with water at 0.5 mL.min$^{-1}$. [b] Retention time was 6.84 min. [c] Stereoregularity deduced from n.m.r. analysis.

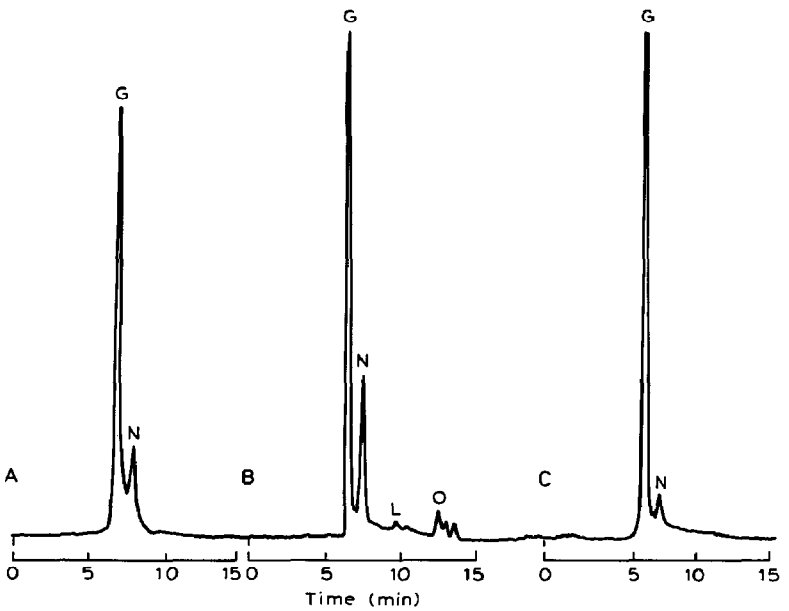


Fig. 1. H.p.l.c. of the products of the action of *C. resinae* endo-(1→3)-α-D-glucanase action on A, *A. niger* (1→3)-α-D-glucan; B, synthetic (1→3)-glucan DBn11; C, products from glucan DBn11 after incubation with (1→3)-β-D-glucanase, exo-(1→3)-α-D-glucanase, and endo-(1→3)-α-D-glucanase: G, glucose; N, nigerose; L, laminaribiose; O, oligosaccharides.

*Action of* C. resinae *endo-(1→3)-α-*D*-glucanase on dextrans from various NRRL strains.* — The enzyme preparation was free from dextranase activity and was suitable for the determination of (1→3;*l*,*c*)-linked units in dextrans. Methylation analysis provided data on the proportion of (1→3;*l*)- and (1→3;*b*)-linked units. The resolution achieved with capillary g.l.c. permitted excellent quantitation of the types of linkage even when these amounted to <0.5% of the total, and the enzymic analysis was equally sensitive and specific. A comparison of the results obtained by the two methods (Table II) revealed a positive relationship, for several soluble (S) and less-soluble (L) dextrans, between the enzymic conversion into D-glucose and the proportion of (1→3;*l*)-linked units determined from mole percentages of 2,4,6-tri-*O*-methyl sugar. There was little or no correlation between enzymic hydrolysis and the proportion of (1→3;*b*)-linked units.

A statistical analysis revealed that the relationship between the data for enzymic ($y$) and methylation analysis ($x$) for eleven NRRL dextrans containing 1–5% of (1→3;*l*)-linked units was rectilinear, with a correlation coefficient ($r$) of 0.887. When the results for two chemically synthesized (1→3)-*α*-D-glucans and one enzymically synthesized (1→3)-*α*-D-glucan were included ($n$ = 14), the value of $r$ rose to 0.999. The equation for the regression line (Fig. 2c) was $y = 0.75x$ (residual standard deviation,

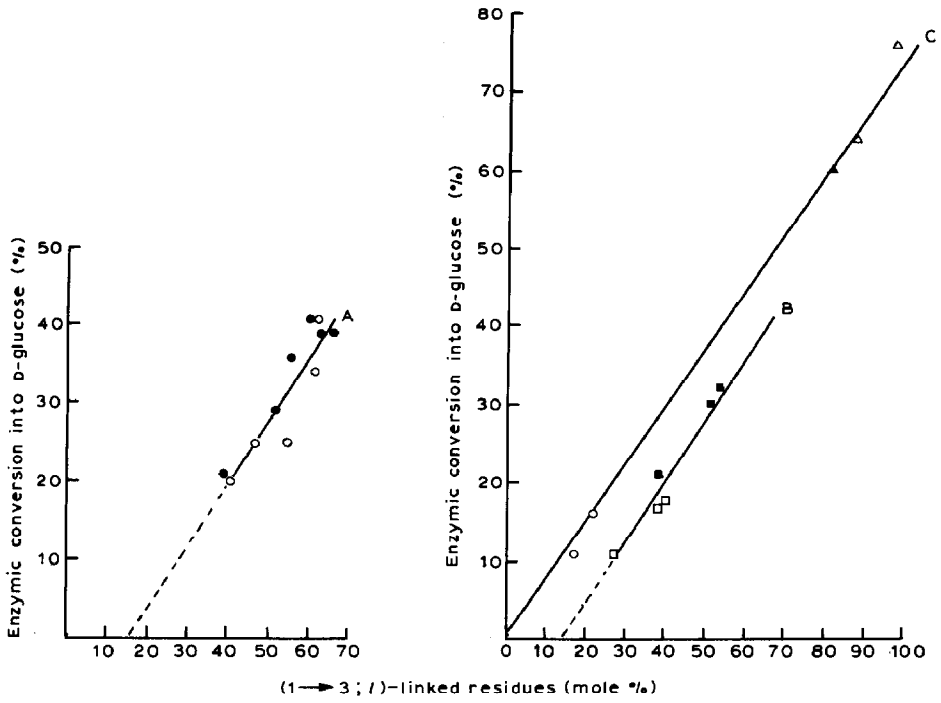


Fig. 2. Relationships between the proportion of (1→3;*l*)-linked residues in dextrans and the limit of their hydrolysis to D-glucose by *C. resinae* endo-(1→3)-*α*-D-glucanase: A, water-insoluble *S. sobrinus* glucans of strains K1-R(O) and OMZ176(●); B, the regression line includes the values in line A together with *S. mutans* strains (■) and unusual dextrans from *Leuconostoc* strains B-523 and B-1149 (□); C, regression line for *Leuconostoc* soluble dextrans (values not shown) and *S. sanguis* dextrans (○), *S. sobrinus* (1→3)-*α*-D-glucan (▲), and synthetic (1→3)-*α*-D-glucans (△).

1.22; coefficient of variation, 7.9%). From these relationships, it is concluded that (1→3;*l*)-linked units in the 11 NRRL dextrans were as accessible to enzymic hydrolysis as those in the synthetic (1→3)-α-D-glucans. The results in Table II also show the extent of solubilization of the dextrans in digests incubated with and without enzyme. In two instances, the enzymic removal of a few percent of (1→3)-linked residues resulted in extensive solubilization of NRRL dextrans (see also Table IV, and Fig. 3).

*Linkage analysis of* S. sobrinus *glucans.* — A series of mixed-linkage α-D-glucans was obtained by growing *S. sobrinus* strain OMZ176 in a chemostat under various defined conditions[19]. The ratio of (1→6) and (1→3) linkages determines the solubility of the glucans, and incubation of cell-free filtrates from each steady state with sucrose provided water-soluble and water-insoluble glucan fractions. The latter contained the higher proportion of sequences of (1→3) linkages. Because the productivity of the D-glucosyltransferase (GTF-I) that catalyzed the synthesis of (1→3)-α-D-glucans was high in culture filtrates obtained (*a*) from glucose-sufficient media and (*b*) from a high rate growth in glucose-limited media, the water-insoluble glucans obtained under these conditions were expected to be the most susceptible to hydrolysis by endo-(1→3)-α-D-

TABLE II

Linkage analysis, enzymic hydrolysis, and solubilization of selected native and synthetic glucans

| *Glucans* | *Fraction or prepn.* | *Linkage analysis* | | *Endo-(1→3)-α-D-glucanase* | | |
|---|---|---|---|---|---|---|
| | | (*1→3*;b) (%) | (*1→3*;l) (%) | *Conversion into glucose* (%) | *Glucan solubilized* (%) | |
| | | | | | *Digest* | *Control* |
| *NRRL dextrans*[a] *B-512(F)*[b] | | 5 | 0 | 0.1 | 100 | 100 |
| 523[c] | S enz.[e] | 3 | 3 | 2.6 | 100 | 100 |
| 1139 | S | 13 | 4 | 1.9 | 100 | 4 |
| 1191[c] | | 19 | 0 | 0.3 | 95 | 93 |
| 1192[c] | | 17 | 1 | 0.5 | 100 | 100 |
| 1298[c] | | 3 | 3 | 2.0 | 100 | 100 |
| 1299[c] | L | 5 | 5 | 5.0 | 94 | 14 |
| 1299[c] | S | 1 | 1 | 0.3 | 100 | 100 |
| 1351[c] | L | 12 | 2 | 0.8 | 100 | 100 |
| 1355[c] | L | 4 | 1.5 | 2.0 | | |
| 1399[c] | S | 1 | 1 | 0.1 | 100 | 100 |
| *S. sobrinus* 6715-13-201 | I enz. | 4 | 82 | 60 | 79 | 0 |
| *Synthetic glucans*[d] | | | | | | |
| DBnll | | 0 | 87.5 | 64 | | |
| DBr26 | | 0 | 97.5 | 76 | | |

[a] NRRL strains are cited in ref. 1. [b,c] For complete results of methylation analysis, see refs. 18 and 15, respectively. [d] These glucans have 100% (1→3,*l*)-linked units; the recorded values are for (1→3,*l*)-α-linked units. [e] Designations (ref. 1) refer to more soluble (S) or less-soluble (L) fractions obtained by precipitation with alcohol from heterogeneous whole culture or enzymic (enz.) dextran preparations; I enz, an enzymically synthesized insoluble glucan.

glucanase. Soluble glucan fractions that contained mainly sequences of (1→6) linkages were expected to be susceptible to hydrolysis by endo-(1→6)-α-D-glucanase, even though dextrans synthesized by *S. sobrinus* dextransucrase (GTF-S1) are highly branched.

The results of methylation analysis and degradation with specific endoglucanases (Table III) confirmed that the water-insoluble glucans contained a higher proportion of (1→3;*l*) than (1→6; *l*)-linked units, whereas the reverse was true for the water-soluble glucans. The regression line obtained by comparing the results of the enzymic conversion into glucose by endo-(1→3)-α-D-glucanase with those of the methylation analysis was rectilinear ($r$ 0.993) and the slope was not significantly different from that obtained with NRRL dextrans and (1→3)-α-D-glucans.

After the digestions with endoglucanases, the glucans became partially soluble (Table III). In contrast to the results with *Leuconostoc* dextrans (Table II), none of the water-insoluble glucans from *S. sobrinus* became completely soluble after treatment with (1→3)-α-D-glucanase (Fig. 3). It was calculated that *S. sobrinus* limit-glucans contained ~25% of (1→3;*l*) linkages that are resistant to enzymic hydrolysis.

Several water-insoluble glucans were also prepared with culture filtrates from *S. sobrinus* strain K1-R obtained under different conditions of growth in the chemostat, and their analyses are given in Table IV. The slope of the regression line relating the results of enzymic hydrolysis to the percentage of (1→3) linkages determined by

TABLE III

Results of methylation analysis and limits of enzymic hydrolysis of water-insoluble and water-soluble glucan fractions synthesized from sucrose by culture filtrates of *S. sobrinus* OMZ176 grown in a chemostat at pH 6.0

| *Growth conditions* | | *Glucan fraction* | | | *Hydrolysis and solubilization of glucans* (%) | | | |
|---|---|---|---|---|---|---|---|---|
| D[a] ($h^{-1}$) | *Glc* (%) | *Sol*[b] | *$Me_3$-Glc* (*mol %*)[c] *2,4,6* | *2,3,4* | *Endo-(1→3)-α-D-glucanase* | | *Endo-(1→6)-α-D-glucanase* | |
| | | | | | *Glc* (%) | *Sol*[d] (%) | *$IM_3$*[e] | *Sol* (%) |
| 0.05 | 0.5 | I | 40 | 18 | 21 | 60 | 8 | 41 |
| 0.05 | 0.5 | S | 9 | 43 | 1 | | 39 | |
| 0.15 | 0.5 | I | 56 | 13 | 36 | 61 | 7 | 35 |
| 0.56 | 0.5 | I | 64 | 11 | 29 | 44 | 10 | 38 |
| 0.56 | 0.5 | S | 22 | 32 | 11 | | 30 | |
| 0.69 | 0.5 | I | 66 | 10 | 39 | 53 | | |
| 0.05 | 4.0 | I | 56 | 12 | 41 | 59 | 3 | 29 |
| 0.05 | 4.0 | S | 5 | 55 | 2 | | 61 | |
| 0.50 | 4.0 | I | 63 | 15 | 39 | 54 | 6 | 17 |
| 0.50 | 4.0 | S | 8 | 62 | 2 | | 72 | |

[a] Dilution rate. [b] I, Water-insoluble; S, water-soluble. [c] Methylation analysis. [d] Solubilization of insoluble glucan. [e] Conversion into apparent isomaltotriose.

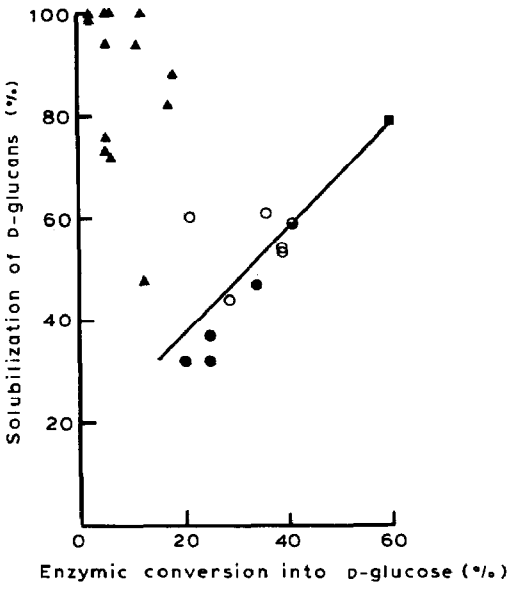


Fig. 3. Effect of degradation with *C. resinae* endo-(1→3)-α-D-glucanase on the solubilization of NRRL dextrans (▲) and glucans from *S. sobrinus* strains OMZ176 (○), K1-R (●), and 6715-13-201 (■). The regression line is for *S. sobrinus* glucans only.

methylation analysis did not differ significantly from that derived from strain OMZ176. Seven water-insoluble dextrans from NRRL strains, not included in the previous analyses (Table II), gave anomalous results for their limit of enzymic hydrolysis (Table IV). Among these strains, B-1149 synthesized dextrans with the highest proportion of (1→3;*l*)-linked units. The co-ordinates for the two dextrans (38,17 and 40,18) were distant from the regression line for the more-soluble *Leuconostoc* dextrans (Fig. 2c), but fitted the strain K1-R line well. The co-ordinates (41,20) for the closest point on the K1-R regression line were for a glucan prepared with culture filtrate obtained at a dilution rate (*D*) of 0.05 $h^{-1}$. Thus, a *Leuconostoc* dextran and a *Streptococcus* water-insoluble glucan, with the same proportion (~40%) of (1→3;*l*)-linked units but with 8 and 22% of branch points, respectively, gave a similar result for the enzymic hydrolysis of their sequences of (1→3) linkages. The B-1149 dextrans became highly soluble (82 and 88%) following the enzymic hydrolysis, and, in this respect, they conformed with the other *Leuconostoc* dextrans (Fig. 3) and not with the *Streptococcus* glucans. The water-insoluble *Leuconostoc* dextrans (Table IV) each had a higher content of (1→3;*l*)-linked units than the more-soluble dextrans listed in Table II, and a substantial proportion remained resistant to endo-(1→3)-α-D-glucanase.

Hydrolysis of the water-soluble glucans of strain K1-R with the endo-(1→3)-α-D-glucanase gave values of 2–8.5% for conversion into glucose, and this range was similar to that obtained with strain-OMZ176 soluble glucans (Table III).

TABLE IV

Analyses of *Leuconostoc* water-insoluble dextrans and comparison with water-insoluble α-D-glucans[a] from *S. mutans* and *S. sobrinus* strain K1-R

| *Source of glucan* | *Methylation analysis Methyl ethers (mole %)* | | | | | *Degradation with endo-(1→3)-α-D-glucanase (%)* | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *2,3,4,6* | *2,4,6* | *2,3,4* | *2,4* | *3,4* | *Glucan solubilized* *Digest* | *Control* | *Conv. to glucose* *Exptl.*[b] | *Calc.*[c] |
| Leuconostoc | | | | | | | | | |
| B-1299 | 29 | 11 | 31 | 3 | 26 | 73 | 4 | 4.6 | 0 |
| B-1433 | 24 | 12 | 39 | 3 | 22 | 72 | 3 | 5.9 | 0 |
| B-1438 | 5 | 17 | 73 | 5 | | 100 | 5 | 5.7 | 4 |
| B-1118 | 4 | 22 | 69 | 5 | | 100 | 20 | 12 | 8 |
| B-1121 | 4 | 23 | 69 | 4 | | 48 | 1 | 12 | 9 |
| B-523[d] | 6 | 27 | 60 | 4 | 3 | 94 | 14 | 11(16) | 12 |
| B-1149[d] | 9 | 38 | 45 | 3 | 5 | 82 | | 17 | 20 |
| B-1149 | 9 | 40 | 44 | 4 | 3 | 88 | 11 | 18 | 21 |
| *S. mutans*[e] | | | | | | | | | |
| NSW47[f] | 12 | 38 | 39 | 11 | | 36 | | 21 | 20 |
| Ingbritt[f] | 7 | 53 | 36 | 4 | | 42 | | 32 | 32 |
| *S. sobrinus*[g] | | | | | | | | | |
| K1-R (a) | 20 | 41 | 17 | 22 | | 32 | 1 | 20(24) | 23 |
| (b) | 19 | 47 | 14 | 20 | | 37 | 2 | 25(31) | 27 |
| (c) | 13 | 55 | 16 | 16 | | 32 | 0 | 25(32) | 33 |
| (d) | 11 | 62 | 13 | 13 | | 47 | 0 | 34(49) | 39 |
| (e) | 12 | 63 | 11 | 13 | | 59 | 1 | 41(52) | 40 |

[a] Prepared by incubating cell-free filtrates with sucrose. [b] Experimental values in parentheses refer to total hydrolysis (glucose plus nigerose). [c] Calculated by allocating 4 mole % of the (1→3;*b*)-linked units to trisubstituted glucose residues in the (1→3)-linked glucan chain, then assuming that four (1→3;*l*)-linked units in the vicinity of these branch points were resistant to enzymic hydrolysis. [d,f] Methylation analyses of *Leuconostoc* and *S. mutans* glucans are from refs. 15 and 4, respectively. [e] Batch cultures. [g] Continuous cultures. The growth conditions were, (a) Glc (4%), pH 6.0, *D* 0.05 $h^{-1}$; (b) Glc (0.5%), pH 7.0, *D* 0.49 $h^{-1}$; (c) Glc (0.5%), pH 7.0, *D* 0.56 $h^{-1}$; (d) Glc (4%), pH 6.0, *D* 0.50 $h^{-1}$; (e) Glc (0.5%), pH 6.4, *D* 0.49 $h^{-1}$.

*Estimation of the proportions of (1→3;1)-linked residues in water-insoluble glucans of various* Streptococcus *sp.* — Water-insoluble glucans were prepared by incubating culture filtrates of other oral streptococci with sucrose. The organisms were grown in batch culture[4] under anaerobic conditions in medium supplemented with 2% of D-glucose, and the pH was maintained at 6.0. Values for the proportion of (1→3;*l*)-linked units in the glucans were calculated from their enzymic hydrolysis with endo-(1→3)-α-D-glucanase, the results for conversion into glucose being read from the regression lines for (*a*) *S. sobrinus* water-insoluble glucans (Fig. 2A) and (*b*) all native glucans (Fig. 4) from *Leuconostoc* sp. and *S. sobrinus* ($n = 34$). This procedure gave values for *S. mutans* glucans that were reasonably close to the experimental values obtained by methylation analysis, but the agreement for the single *S. salivarius* glucan was only fair. For *S. sanguis* dextrans, which are more closely related to the *Leuconostoc* dextrans, it was necessary to use the regression line for all native dextrans in order to make a satisfactory estimate (Table V).

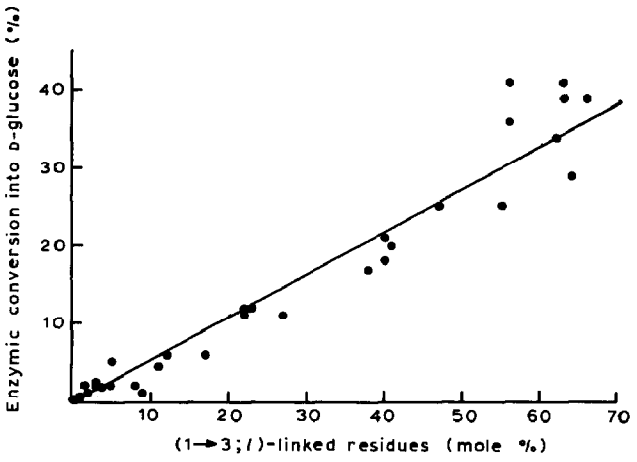


Fig. 4. Regression line for soluble and water-insoluble *Leuconostoc* dextrans and *S. sobrinus* native glucans. The equation was $y = 0.578\ x$.

TABLE V

Estimation of (1→3;*l*)-linked residues in water-insoluble glucans of oral streptococci from their enzymic conversion into D-glucose

| *Species* | *Conv. into glucose* (%) | *Linkage analysis (1→3;l)* | | |
|---|---|---|---|---|
| | | *Calc. from regression equation for* | | *Methylation analysis* |
| | | S. sobrinus (%) | *All native dextrans* (%) | *2,4,6-Me₃-Glc (mole %)* |
| *S. mutans* | | | | |
| Ingbritt | 32 | 55 | 57 | 53 |
| NSW1 | 30 | 53 | 54 | 51 |
| NSW47 | 21 | 41 | 38 | 38 |
| *S. salivarius* | | | | |
| ATCC 13419 | 30 | 53 | 54 | 59 |
| *S. sanguis* | | | | |
| 804 | 13 | 31 | 23 | 21 |
| 10558 | 11 | 29 | 19 | 17 |

*Analysis of three soluble dextrans from* S. sobrinus *strains.* — The soluble glucan synthesized by an extracellular glucosyltransferase (formerly GTF-S, now GTF-S1), separated from culture filtrates of *S. sobrinus* OMZ176, is a highly branched dextran. Methylation analysis[4] and incubation with *C. resinae* endo-(1→3)-α-D-glucanase[4,7] confirmed the absence of (1→3;*l*)-linked units in this dextran (S1-dextran). Two more glucosyltransferases (GTF-S3 and GTF-S4) were released when *S. sobrinus* strains were

TABLE VI

Linkage analysis of *S. sobrinus* soluble dextrans synthesized with glucosyltransferases (GTF) separated from culture filtrates

| *Strain* | *GTF*[a] | *Hydrolysis of dextran (%)* | | *Methyl ethers (mol %)* | | | |
|---|---|---|---|---|---|---|---|
| | | *Endo-(1→3)-α-D-glucanase* | *Endo-(1→6)-α-D-glucanase*[b] | *2,3,4,6* | *2,4,6* | *2,3,4* | *2,4* |
| K1-R | S1[c] | 0.3 | 45 | 33 | tr | 35 | 32 |
| 6715-13-210 | S1 | 0.7 | 27 | 32 | | 34 | 33 |
| 6715-13-27 | S1 | | 29 | 35 | tr | 30 | 35 |
| K1-R | S3[d] | 0.7 | 123 | 5 | | 95 | |
| 6715-13-201 | S3 | 0.3 | 101 | 5 | | 95 | |
| K1-R | S4[e] | 13.5 | 85 | 7 | 19 | 62 | 12 |
| 6715-13-201 | S4 | 10.2 | 84 | 9 | 17 | 64 | 10 |
| 6715-13-27 | S4 | 5.4 | 97 | 8 | 6 | 77 | 8 |
| | S4[f] | | 105 | 7 | 2 | 85 | 6 |

[a] Isolated by chromatography on hydroxyapatite. [b] From *Penicillium funiculosum*. [c–e] Incubation of GTF-S1, -S3, and -S4 with sucrose produced S1-dextran, S3-dextran, and S4-dextran, respectively. [f] Additional chromatography on Trisacryl-DEAE was used.

grown in medium supplemented with Tween 80. The mutant strain 6715-13-27 was able to release GTF-S4 in the absence of Tween 80.

The relationship between the results of enzymic hydrolysis and methylation analysis for S1-, S3-, and S4-dextran from the three strains of *S. sobrinus* is shown in Table VI. Each of the S1-dextrans was highly branched, and (1→3;*l*)-linked units were virtually absent. The two methods of analysis established that neither (1→3;*l*)- nor (1→3;*b*)-linked units were present in the S3-dextrans. In contrast, the S4-dextran preparations from strains K1-R and 6715-13-201 contained both (1→3;*l*)- and (1→3;*b*)-linked units, with the former accounting for ~18% of the total. Mutant 27 GTF-S4 synthesized a dextran with a similar content of branch points, but the molar proportion of 2,4,6-tri-*O*-methylglucose was reduced to 6%. These differences between the S4-dextrans from three strains could result from the incomplete separation of GTF-I, the glucosyltransferase that synthesizes (1→3)-*α*-D-glucan, from the GTF-S4 preparations. Strain 6715-13-27 is a mutant that is deficient in GTF-I production[20]. Enzyme from an additional purification step, designed to remove traces of GTF-I from the GTF-S4 preparation, synthesized a dextran containing <2% of (1→3;*l*)-linked units. These results suggest that purified preparations of the three GTF-S types from *S. sobrinus* synthesize three different dextrans with virtually no (1→3;*l*)-linked units.

## DISCUSSION

Although endoglucanases generally fragment glucans to give a characteristic distribution of oligosaccharides, *C. resinae* endo-(1→3)-α-D-glucanase is representative of a group of fungal enzymes that hydrolyze water-insoluble (1→3)-α-D-glucans from fungal cell walls to give mainly D-glucose[11,21,22]. In some digests, traces of nigerose were present. Incubation of *C. resinae* endo-(1→3)-α-D-glucanase with water-insoluble glucans from batch-grown oral streptococci, followed by p.c. of the products, revealed that the sequences of (1→3) linkages had been hydrolyzed to give an appreciable yield of nigerose together with D-glucose[4]. In the present study, the products from several synthetic and microbial (1→3)-α-D-glucans were fractionated by h.p.l.c., and the weight ratio of D-glucose to nigerose was found to be ~4:1.

The recent chemical syntheses of linear (1→3)-D-glucans has provided substrates free from branches or other linkages. They do not contain the (1→4;*l*)-linked residues[23] that have been detected in some preparations of native (1→3)-D-glucans. The high degree of stereoregularity of the best preparations of synthetic (1→3)-D-glucans is shown by their enzymic hydrolysis to give >97% of products derived from α-linked sequences (Fig. 1). The release of the β-(1→3)-linked disaccharide, laminaribiose, proved that *C. resinae* endo-(1→3)-α-D-glucanase was able to hydrolyze α-(1→3) linkages adjacent to the β residues that were present in minor amounts in the glucans. The 3-α-laminaribiosylglucose and 3-α-glucosyl-laminaribiose were most likely released through the hydrolysis of α-(1→3) linkages further removed from β residues. Such trisaccharides would be resistant to hydrolysis because the enzyme requires substrates that contain a minimum of two contiguous α-(1→3)-linked residues. Nigerotriose, the smallest substrate for *C. resinae* endo-(1→3)-α-D-glucanase, is slowly hydrolyzed to glucose and nigerose[7]. The enzyme does not hydrolyze isolated (1→3) linkages in such oligosaccharides as $3^3$-glucosylisomaltotriose, or in such polysaccharides as isolichenan, nigeran, or dextrans having alternating (1→3)- and (1→6)-linked residues. Hence, the appearance of αβ- and βα-glucotrioses as stable products is consistent with the known specificity of the endoglucanase.

In several *Leuconostoc* dextrans that contain 1–5% of (1→3;*l*)-linked units, good agreement was found between the mole percent of (1→3)-linked residues and the release of D-glucose by enzymic hydrolysis (Table II). Therefore, these dextrans must contain at least two contiguous (1→3)-linked residues incorporated into the repeating unit, and these could occur as side chains containing two or more (1→3)-linked residues. In such locations, the (1→3)-linked residues would be in peripheral positions, where maximal accessibility to the enzyme would be assured. The rectilinear relationship between the content of (1→3;*l*)-linked units and enzymic conversion into D-glucose for the *Leuconostoc* dextrans, *Streptococcus sobrinus* (1→3)-α-D-glucan, and chemically synthesized (1→3)-D-glucans (Fig. 2c) indicated that the (1→3) linkages in each of these substrates were available equally for enzymic hydrolysis.

Many of the less-soluble NRRL dextrans became water-soluble after incubation with endo-(1→3)-α-D-glucanase, even when there was only 2–5% hydrolysis to glucose

(Fig. 3). This effect indicated that the sequences of (1→3) linkages, although a minor structural feature, were responsible for the insolubility of the dextrans. Bourne *et al.*[24] suggested that the soluble (S) fractions and insoluble (I) or less (L) soluble fractions of strain B-1299 dextran represented successive stages in the synthesis, the latter stage being the addition of (1→3)-linked residues to give dextran L. We have now demonstrated the reverse effect, namely the hydrolysis of the extra (1→3) linkages in B-1299 dextrans I and L to give soluble dextran. A peripheral location for the (1→3;*l*)-linked units in B-1299 dextran fraction L has been deduced from several studies, and there is evidence that some nigerosyl groups are attached to the B-1299 dextran (fraction S) chain by (1→2;*b*)-linked units[24,25]. It is significant that nigerotriose and nigerotetraose have been isolated from acetolysates[26] of B-1299 dextran (fraction I). A nigerotriosyl side-chain would be susceptible to hydrolysis by this endoglucanase. The enzyme may also recognise a nigerosyl side-chain attached to the main chain by a (1→3;*b*)-linked unit and release a single glucose residue.

It has long been known that the (1→3) linkages in water-insoluble glucans of *Streptococcus sobrinus* are synthesized by a D-glucosyltransferase (GTF-I) that converts sucrose into a (1→3)-*α*-D-glucan[3,4,22]. The (1→3)-glucan chains in the mixed-linkage glucans are therefore also linear and contain contiguous units designated (1→3;*l,c*). However, *C. resinae* endo-(1→3)-*α*-D-glucanase did not hydrolyse all the (1→3;*l*)-linked units shown to be present by methylation analysis.

Since (1→3;*nc*)-linked units are unlikely to be found in these glucans, the resistant linkages most probably involve (1→3;*l,c*)-linked units that are located in the vicinity of the branch points. The glucans may contain three types of branch points. The dextran-like regions would have the same (1→3;*b*)-linked units branched at positions 6 that are found in the S1-dextrans (Table VI) synthesized by the glucosyltransferase designated GTF-S1. The dextran chains may also bear (1→3)-glucan chains that are attached by means of (1→3;*b*)-linked units through positions 6. In addition, there is evidence[27] that the (1→3)-glucan chains bear dextran side-chains attached via (1→6;*b*)-linked units branched at positions 3. By analogy with branched dextrans, where two linkages either side of a branch point can be resistant to hydrolysis by endodextranase[28], it can reasonably be assumed that, for *S. sobrinus* water-insoluble glucans, in regions of the (1→3)-linked glucan chains where highly branched dextran-type chains are attached, there must be several (1→3;*l,c*) linkages that cannot be hydrolyzed by endo-(1→3)-*α*-D-glucanase. This view is consistent with the severe effect on the limit of hydrolysis of (1→3)-*α*-D-glucan brought about by the introduction of a few carboxymethyl groups.

Determination of mole percentages of 2,4-di-*O*-methylglucose by methylation analysis can only give a total value for branch points in *S. sobrinus* glucans, and, at present, it is difficult to estimate the relative distribution of the three possible types. An assessment of the most probable proportion of inter-chain linkages has been made by comparing the results of analyses of *S. sobrinus* glucans with those of unusual *Leuconostoc* B-523 and B-1149 dextrans that contain a high proportion of (1→3;*l,c*)-linked residues (Table IV). The *Leuconostoc* dextrans contain some 4% of (1→3,6)-branch points. It is assumed that each of these represents an inter-chain linkage, and that there

are four resistant (1→3) linkages for each branch point. On this basis, the estimated enzymic conversion into glucose was in good agreement with that determined experimentally.

Two of the *Streptococcus* glucans shown in Table IV, namely, those from *S. mutans* NSW47 and *S. sobrinus* K1-R, contain a proportion of (1→3;*l*)-linked units similar to that found in B-1149 dextran. The values for 2,4,6-tri-*O*-methylglucose ranged from 38–41 mole % and the three glucans were hydrolyzed by *C. resinae* endo-(1→3)-α-D-glucanase to a similar extent (range 17–21% conversion into D-glucose). This result indicated that all three contained a similar proportion of resistant (1→3;*l*,*c*)-linked units. Accordingly, it is deduced that, in *Streptococcus* glucans, the (1→3)-linked chains contain the same number of branch points as the unusual *Leuconostoc* dextrans, namely, 4%. When the proportions of resistant (1→3;*l*,*c*)-linked units in the other *S. sobrinus*, *Leuconostoc*, and *S. mutans* glucans were calculated on this basis, the estimated conversions into glucose agreed well with the experimentally determined values (Table IV).

Insoluble dextrans from *Leuconostoc* strains B-523 and B-1149 have been grouped with *S. sobrinus* water-insoluble glucans[2,5] because they display $^{13}C$ resonances that are diagnostic for (1→3)-linked residues. They also give a significant i.r. band at 822 $cm^{-1}$, which is characteristic of moderate to large proportions of (1→3;*l*,*c*)-linked units. When the results of enzymic hydrolysis and methylation analysis of the (1→3;*l*)-linked units in the unusual *Leuconostoc* dextrans were added to those for the water-insoluble glucans from *S. sobrinus* strains K1-R and OMZ176, the correlation coefficient for the regression line was raised from 0.91 to 0.95.

The D-glucosyltransferases of *Leuconostoc* and *Streptococcus* probably operate in a similar fashion. Thus, although little is known about the relative positions of the (1→3;*l*,*c*)-linked units and the linear (unbranched) dextran-like regions of B-1149 insoluble dextran, analogy with *S. sobrinus* glucans would indicate that the two types of chain are linked. The most obvious difference between glucans from the two genera (Table IV) is the high degree of linearity of many of the *Leuconostoc* dextrans compared with that of *S. sobrinus* glucans. However, in one model[27] proposed for the water-insoluble glucan from *S. sobrinus* OMZ176, *all* the branch points were (1→6;*b*)-linked units that connected short, linear dextran chains, and single glucosyl residues, to a (1→3)-glucan main chain, with the average number of residues between branch points being 3–4. The release of glucose by enzymic hydrolysis of such a highly branched (1→3)-α-D-glucan is not compatible with the specificity of *C. resinae* endo-(1→3)-α-D-glucanase. In contrast, the structure proposed for *S. sobrinus* glucans as a result of the present study can be reconciled with the limits of hydrolysis of several glucans derived from different streptococci grown under various conditions. For instance, the hydrolysis predicted for water-insoluble *S. sobrinus* OMZ176 glucan obtained at high rate of growth in excess of D-glucose would be $0.78 \times (63 + 4 - 16) = 40\%$, which is in good agreement with the value (39%) obtained experimentally (Table III). The enzymic analyses also support the basic structure, which contained branched dextran chains and more-linear (1→3)-α-D-glucan, proposed previously[4]. The dextran-like regions may be

peripheral to regions where the sequences of (1→3)-linked units are located, with some 14–16% of the (1→3;*l*,*c*)-linked units in the less accessible inner regions protected from enzymic hydrolysis. Thus, the limit glucans remaining after hydrolysis of the water-insoluble glucans would retain a sufficient proportion of sequences of (1→3)-linked units to prevent their solubilization.

The minor amounts of (1→3;*l*)-linked units that occur in *S. sobrinus* soluble-glucan fractions are also resistant to enzymic attack (Table III). By contrast, the low proportions of (1→3;*l*)-linked units found in some *Leuconostoc* dextrans (Table II) are located in shorter, peripheral chains, which are available for enzymic hydrolysis. The unexpectedly strong *α*-(1→3)-immunoactivity of B-1299 dextran S has been cited[29] as evidence to support a peripheral location for nigerosaccharide groups. Hydrolysis of such groups may account for most of the glucose released from B-1299 and B-1433 water-insoluble dextrans (Table IV). Half the (1→3;*l*)-linked units in these highly branched dextrans were inaccessible to the enzyme, and 28% of the limit dextrans remained water-insoluble.

*Analysis of water-insoluble glucans from batch-grown oral streptococci.* — The use of regression lines for estimating the proportion of (1→3;*l*,*c*)-linked residues in glucans from other *Streptococcus* spp (Table V) was tested. When the line for *S. sobrinus* insoluble glucans was used (Fig. 2A), the limit of enzymic hydrolysis of *S. mutans* glucans provided values that agreed well with those known from methylation analysis. This agreement suggested that, among the mutans group of streptococci, strains of *S. mutans* and *S. sobrinus* produced water-insoluble glucans with similar structures. The procedure failed to give a satisfactory estimate for (1→3;*l*,*c*)-linked units in *S. sanguis* dextrans. These dextrans differ from the insoluble dextrans of the mutans streptococci, as shown by their low limit of hydrolysis by *C. resinae* endo-(1→3)-*α*-D-glucanase, and differ from the insoluble *Leuconostoc* dextrans by the absence of the characteristic i.r. band at 822 $cm^{-1}$ in *S. sanguis* 804 dextran[30]. A more reliable estimate of the (1→3;*l*)-linked units in *S. sanguis* dextrans was obtained using a regression line for all native dextrans (Fig. 4).

A comparison, by covariance analysis, of the individual regression lines for the *Leuconostoc* dextrans listed in Table II and *S. sobrinus* glucans (Fig. 2A) with the common regression line for native dextrans (Fig. 4) showed no significant difference between the slopes of the lines. The high value (0.973) of the correlation coefficient for the common regression line makes it suitable for general use with dextrans of unknown structure. When the values for three synthetic glucans (Table II) having 82–98% of (1→3;*l*)-linked units were included in the analysis ($n = 37$), the regression became significantly curvilinear. The equation was $y = 0.2672x + 0.0051x^2 + 0.89$, with multiple correlation coefficient R = 0.986 and F = 30.9 ($P < 0.01$). For (1→3)-*α*-D-glucan (where $x$ is 100%) the value for $y$, the conversion into D-glucose, was 78%, in excellent agreement with the values found experimentally. It appears that simple determinations of enzymic conversion of the dextrans into D-glucose can provide estimates of the proportion of (1→3;*l*,*c*)-linked residues that are more reliable and more sensitive than those deduced from F.t.-i.r. difference-spectra[5] or from $^{13}C$-n.m.r. spectroscopy[2].

*Analysis of* S. sobrinus *soluble glucans.* — Unfractionated culture filtrates of *S. sobrinus* can contain up to four glucosyltransferases which react with sucrose to produce a soluble dextran fraction and a water-insoluble glucan. The soluble dextran fraction from strain OMZ176 contained 5–22 mole % of (1→3;*l*)-linked residues. Separation of three different GTF-S enzymes from *S. sobrinus* culture filtrates has given at least two glucosyltransferases that synthesize dextrans containing no (1→3;*l*)-linked residues (Table VI). The third enzyme, GTF-S4, after more extensive purification, also synthesized a dextran containing few (1→3;*l*)-linked units (2% or less). These results imply that the only *S. sobrinus* enzyme capable of synthesizing (1→3;*l*,*c*)-linked residues is GTF-I. Therefore, preparations of *S. sobrinus* glucosyltransferases that synthesize soluble dextrans containing 25–28% of (1→3;*l*)-linked units[31,32] are likely to contain unresolved mixtures of GTF-S and GTF-I.

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