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# OXIDIZED SACCHARIDES AS INHIBITORS OF α-GLUCAN SYNTHESIS BY *Streptococcus mutans* GLUCOSYLTRANSFERASE

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ABSTRACT

Specific inhibition by periodate-oxidized dextrans of the synthesis of α-glucan by *S. mutans* glucosyltransferase prompted a search for structurally related inhibitors that might be effective as anticaries agents. Clinical dextran derivatives in which from 5 to 50% of the D-glucose units were oxidized acted as potent and specific enzyme-inhibitors, as did 10%-oxidized derivatives of dextran fractions ranging in mol. wt. from $10^4$ to $2 \times 10^6$. Within these limits, differences in oxidation or molecular weight did not significantly affect the high inhibitory potency of the derivatives. In contrast, periodate oxidation of (1→6)-α-, (1→3)-α-, and (1→4)-α-linked oligosaccharides containing $< \sim 15$ D-glucose units, and of sucrose and structurally related trisaccharides, yielded derivatives that were poor inhibitors. Enzymic hydrolysis of oxidized dextrans caused a loss of their inhibitory power and indicated that, to act as specific inhibitors, oxidized molecules must contain at least 16 to 20 D-glucosyl residues. The similar, minimum size required in order that unoxidized oligosaccharides may act as efficient acceptors in the glucosyltransferase reaction suggests that the inhibitory potencies of oxidized derivatives may reflect their relative abilities to bind at the acceptor site of the enzyme.

## INTRODUCTION

The occurrence of dental plaque and carious lesions has been related to the synthesis of α-glucans from dietary sucrose by extracellular enzymes of oral streptococci[1–4]. *Streptococcus mutans* strains isolated from carious lesions[5] produce extracellular, water-insoluble α-glucans that enable the bacterial cells to adhere tenaciously to smooth, tooth surfaces[6–8], and so contribute to their high cariogenic potential. The α-glucans are synthesized by glucosyltransferases (EC 2.4.1.5) that polymerize the D-glucosyl group of sucrose, with the release of D-fructose. Studies on glycosyltransferases of *S. mutans* have been reviewed by Montville *et al.*[9].

We have described[10] the action of partially oxidized dextran as a potent and

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Clinical dextran (1 g) was also partially hydrolyzed with 0.5M sulfuric acid (100 mL) for 2 h at 100°, the hydrolyzate cooled to 0°, the acid neutralized with barium carbonate, and the suspension filtered. Solutions of the oligosaccharides obtained by acetolysis, or acid hydrolysis, were de-ionized with AG-50 1-X8 CD (carbonate) mixed-bed resin, concentrated under diminished pressure, and applied to a column of Bio-Gel P-2. Oligosaccharides in the eluted fractions were detected by thin-layer chromatography on plates of silica gel, and fractions were so combined as to give solutions containing oligosaccharides of a known range of molecular sizes.

*Periodate oxidation of carbohydrates.* — Polysaccharide or oligosaccharide solutions (10 mg/mL) were treated with the required molar proportions of sodium metaperiodate, as previously described[10]. Complete oxidation of clinical dextran in the presence of an excess of the oxidant resulted in the consumption of two molar proportions of periodate (1.96) per D-glucosyl residue. All of the periodate was consumed in mixtures in which the molar proportion of oxidant and D-glucosyl residues was equal to, or less than, two.

After addition of ethylene glycol to decompose the excess of periodate, solutions containing oxidized polysaccharides were dialyzed against distilled water, and lyophilized. Solutions containing oxidized oligosaccharides were passed through columns of Bio-Rad AG-50 1-X8 CD (carbonate) mixed-bed resin, and lyophilized. For some samples of the completely oxidized oligosaccharides, ~20% was not recovered from the mixed-bed resin, but there was no loss of partially oxidized oligosaccharides. Before use, the oxidized carbohydrates were dissolved, at appropriate concentrations, in 0.02% sodium azide solution, and the solutions were heated for 15 min at 90°, in order to activate them[10]. For convenience, values of per cent oxidation of dextran given in the results are based on the molar proportions of periodate and D-glucosyl residues in the oxidation mixtures. However, it cannot be assumed that an equimolar proportion of periodate (100% oxidation) oxidizes all of the D-glucosyl residues, because a number of them are probably oxidized by more than one periodate ion, even at low molar proportions of the oxidant[14].

*Enzymic hydrolysis of periodate-oxidized dextran.* — Oxidized-dextran preparations (18 mg/mL) were incubated at 37° and pH 6.0 with dextranase (0.1–2.0 international units/mL) in digests containing 50mM sodium phosphate and 0.02% of sodium azide. The release of copper-reducing sugars was measured by the Nelson procedure, and the reaction was terminated at the required extent of hydrolysis by heating the reaction mixture for 10 min at 100°.

Samples (108 mg) of unoxidized and 10%-oxidized, clinical dextrans that had been ~6% hydrolyzed by the action of dextranase were applied to a column (92 × 2.6 cm) of Bio-Gel P-6 at 25°. The column was irrigated with 50mM sodium phosphate solution, pH 7.5, containing 0.02% of sodium azide. The flow-rate was ~25 mL/h and 3-mL fractions were collected. An identical column was calibrated with a mixture of the (1→4)-α-D-glucan chains of debranched glycogen. The average length of the D-glucan chains eluted in fractions was determined from the ratio of total D-glucose

equivalents (phenol–sulfuric acid method) to reducing D-glucose equivalents (Nelson method) present.

*Propagation of cells.* — *S. mutans* cells were grown for 18 h at 37° under 19:1 $N_2$–$CO_2$ in Trypticase-yeast extract broth, pH 7.0, containing cysteine hydrochloride (50 mg/L) and supplemented with D-glucose (10 g/L) as the main carbon-source. The medium was inoculated with stationary-phase cells (10% by volume) maintained in the same broth containing solid $CaCO_3$ to prolong the cell viability. Cells were harvested by centrifugation at 13,000*g* for 15 min at 4°.

*Preparation of extracellular glucosyltransferase.* — Cell-free medium from D-glucose-supplemented cultures of *S. mutans* 6715-49 was brought to 50% saturation at 4° by addition of solid ammonium sulfate. The precipitate was kept in suspension for 4 h at 4°, recovered by centrifugation at 13,000*g* for 20 min, and the washed precipitate was resuspended in 50mM sodium phosphate–0.02% sodium azide solution, pH 6.0 (2.5% of the original culture–volume). The suspension was dispersed by several strokes in a glass homogenizer, and dialyzed at 4° against several changes of the same buffer solution. A small amount of insoluble material was removed by centrifugation. The specific activity of glucosyltransferase in a typical, ammonium sulfate fraction was ~0.03 international units/mg of protein.

A portion of the (ammonium sulfate) enzyme fraction was applied to a column of Sephadex G-50, and the bound-enzyme activity was eluted with 6M guanidine hydrochloride solution, pH 6.0, as previously described[15]. Guanidine hydrochloride was removed by dialysis against 0.02% sodium azide solution, and fractions containing glucosyltransferase activity were combined (specific activity, 3.5–6.1). A portion of this enzyme solution was adsorbed to an ethylamine–Sepharose column, and the enzyme activity was released with a concentration gradient of sodium chloride. This enzyme purification gave a specific activity of 10–13.5.

The three enzyme-fractions used in these studies are referred to as the ammonium sulfate-, guanidine-, and ethylamine-glucosyltransferase preparations. Small proportions of dextranase and invertase were present in the ammonium sulfate-enzyme, but none was detected in the guanidine and ethylamine preparations. Tests with sucrose specifically radiolabelled in either the D-glucosyl or D-fructosyl group established that fructosyltransferase was not present in any of the enzyme preparations. [U-$^{14}$C]Sucrose was therefore used routinely in all enzyme assays.

*Assay of α-glucan synthesis by* S. mutans *glucosyltransferase.* — Enzyme was incubated at 37° with $^{14}$C-sucrose (2.1 mg, 50 nCi) and clinical dextran (150 μg) in a reaction mixture (150 μL) containing 0.02% of sodium azide and 50mM sodium phosphate at pH 6.0. After 15 or 30 min, duplicate samples (50 μL) were removed, and spotted on squares of filter paper, and the $^{14}$C-glucose incorporated into the ethanol-insoluble dextran was measured as previously described[16]. One unit of glucosyltransferase is that amount of enzyme that catalyzes the incorporation of 1 μmol of D-glucose into glucan in 1 min under the foregoing conditions of assay.

*Determination of enzyme inhibition.* — Glucosyltransferase was preincubated at 37° with different amounts of oxidized polysaccharide or oligosaccharide in a

solution (100 μL) containing 50mM sodium phosphate, pH 6.0. After a suitable time of preincubation at 37°, substrate solution (50 μL) containing $^{14}$C-sucrose (2 mg) and clinical dextran (150 μg) were added, and the reaction mixtures (150 μL) were incubated for 15 or 30 min at 37°. Samples (50 μL) were removed, and spotted on squares of filter paper, to permit measurement of the $^{14}$C-glucose incorporated into the ethanol-insoluble polysaccharide. Enzyme inhibition is expressed as percent loss of activity compared to enzyme preincubated with buffer solution or unoxidized, clinical dextran.

*General methods.* — Polysaccharide and oligosaccharide concentrations were determined by the phenol–sulfuric acid method[17], and the release of reducing sugars was measured by a modified Nelson procedure[18]. Protein concentration was determined as described by Lowry *et al.*[19]. Thin-layer chromatography of oligosaccharides was conducted on plates of silica gel as described by Huber *et al.*[20].

## RESULTS

*Inhibition of glucosyltransferase by 10%-oxidized, clinical dextran.* — Some ammonium sulfate-glucosyltransferase preparations were exceptionally susceptible to inhibition by 10%-oxidized dextran. Preincubation of enzyme for 10 min with 10%-oxidized dextran (17 μg/mL) decreased the synthesis of α-glucan by 75%, and pre-

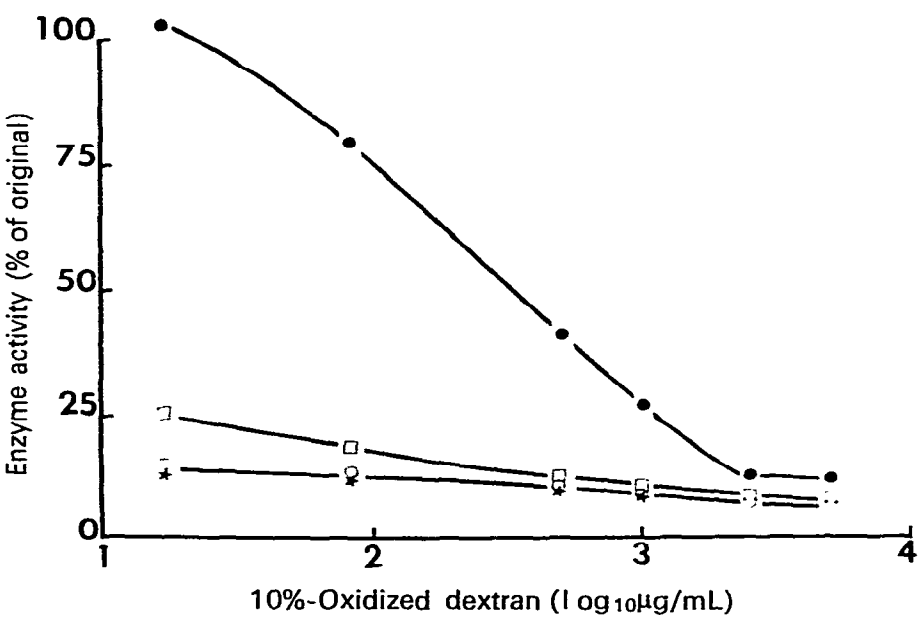


Fig. 1. Effect, on glucosyltransferase activity, of pre-incubation for different times with different concentrations of 10%-oxidized, clinical dextran. [Ammonium sulfate-enzyme (0.6 units/mL) was preincubated for 0 (●), 10 (□), 30 (○), and 60 min (★) with the indicated concentrations of 10%-oxidized dextran, and assayed as described in Materials and Methods.]

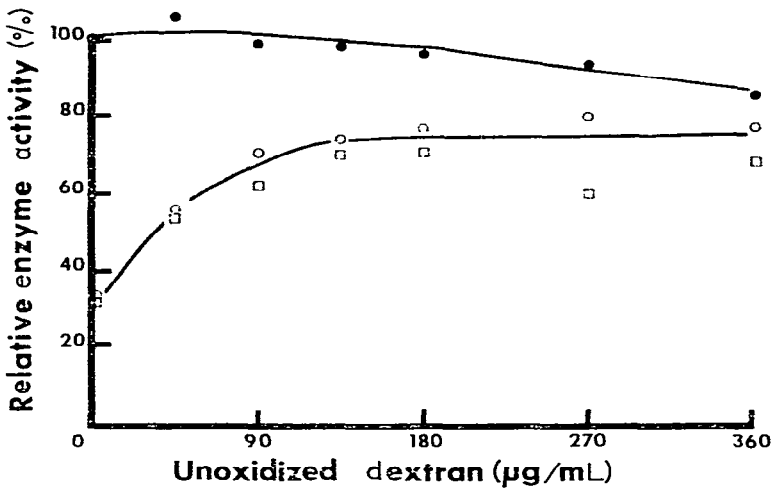


Fig. 2. Effect of the presence of unoxidized, clinical dextran on the inhibition of glucosyltransferase by 10%-oxidized, clinical dextran. [Ammonium sulfate-enzyme (0.3 unit/mL) was pre-incubated for 20 min with (90 μg/mL, ○; 180 μg/mL, □), and without (●) 10%-oxidized dextran, in the presence of the indicated concentrations of unoxidized clinical dextran (abscissa). Preincubation conditions and assay of enzyme activities after preincubation were as described in Materials and Methods.]

incubation for longer periods and with higher concentrations of oxidized dextran resulted in a 90% decrease in glucan synthesis (see Fig. 1). Synthesis was not decreased when the enzyme was preincubated alone, or with unoxidized dextran (not shown). Glucan synthesis by this enzyme preparation was stimulated about twenty-fold when unoxidized dextran was added to the reaction mixture.

However, glucan synthesis by most ammonium sulfate-enzyme preparations was inhibited up to 50–80% by preincubation with 10%-oxidized dextran, and was stimulated only about five-fold by addition of unoxidized dextran to the enzyme reaction-mixture. When 45 μg of clinical dextran was added to enzyme preincubation mixtures (1 mL) containing 10%-oxidized dextran (90 and 180 μg), inhibition was decreased from ~70 to 45%, and 360 μg of clinical dextran afforded the enzyme almost complete protection from inhibition (see Fig. 2).

The time–reaction progress curve of the synthesis of D-glucan catalyzed by the residual activity (40% of the original) of glucosyltransferase preincubated (20 min) with 10%-oxidized dextran (100 μg/mL), and then incubated with sucrose, was identical to that obtained when uninhibited enzyme was diluted to 40% of the original activity and preincubated with unoxidized clinical dextran (100 μg/mL). In the absence of any exogenous dextran, the amounts of glucan synthesized by undiluted and diluted solutions of uninhibited enzyme were, respectively, 50 and 90% lower.

Glucan synthesis by most ammonium sulfate-enzyme preparations, and by all guanidine-enzyme preparations, was inhibited proportionately more (50–80%) by pre-incubation with 10%-oxidized dextran than was the release of copper-reducing sugars (30–50%) from sucrose. However, the absolute decreases of glucan synthesis

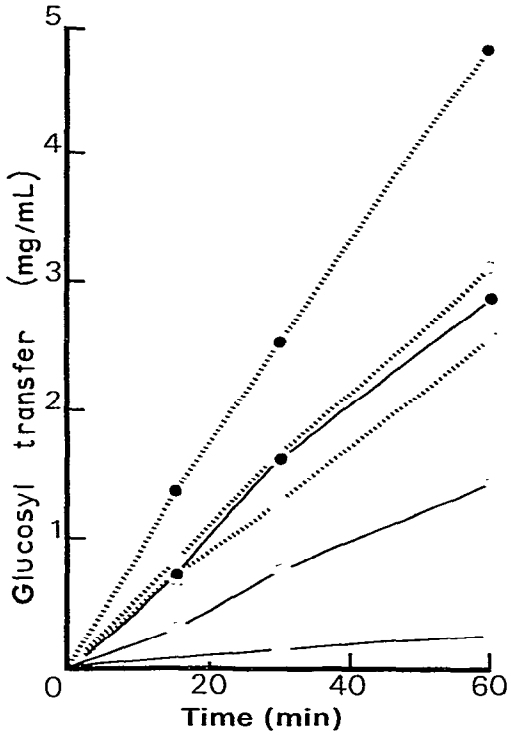


Fig. 3. α-Glucan synthesis, and release of reducing sugars, in the presence and absence of acceptor dextran by glucosyltransferase pre-incubated with 10%-oxidised dextran. [Guanidine-enzyme (0.3 unit/mL) was pre-incubated for 20 min with (○) and without (●, □) 10%-oxidized dextran (100 g/mL). $^{14}$C-Sucrose solution with (●, ○) and without (□) acceptor dextran was added to each reaction mixture. α-Glucan synthesis (solid lines) and release of reducing sugars (dotted lines) were measured at the times indicated. All conditions and assays were as described in Materials and Methods.]

and reducing-sugar release were not greatly different; the apparent disparity in inhibition resulted from the substantially higher release of reducing sugars than could be accounted for by the synthesis of ethanol-insoluble glucan (see Fig. 3).

*Inhibition of glucosyltransferase by other oxidized dextrans.* — Clinical dextran derivatives, in which from 5 to 50% of the D-glucosyl residues were oxidized, inhibited glucan synthesis by glucosyltransferase to about the same extent, but 1%- and 100%-oxidized dextrans were much poorer inhibitors, especially at low concentrations (see Table I). Ammonium sulfate- and guanidine-enzyme preparations were equally inhibited, but ethylamine-enzyme was less inhibited. Different batches of enzyme differed in susceptibility to inhibition by 100%-oxidized dextran. As a rule, enzyme preparations highly susceptible to inhibition by 10%-oxidized dextran (see Fig. 1) were inhibited to a significant extent by 100%-oxidized dextran (see Fig. 4), but none of the enzyme preparations were specifically inhibited by fully oxidized (200%) dextran in which all of the D-glucosyl residues are oxidized. High concentra-

TABLE I

INHIBITION OF GLUCOSYLTRANSFERASE BY DEXTRAN SUBJECTED TO DIFFERENT DEGREES OF PERIODATE OXIDATION

| *Degree of oxidation of dextran (%)* | *Inhibition (%) of glucosyltransferase preparations[a] after preincubation with different amounts of the oxidized dextrans* | | | | | |
|---|---|---|---|---|---|---|
| | *13 μg/mL* | | *65 μg/mL* | | *325 μg/mL* | |
| | *A* | *C* | *A* | *C* | *A* | *C* |
| 1 | 3 | 1 | 17 | 9 | 29 | 8 |
| 5 | 44 | | 55 | | 63 | |
| 7 | 47 | 26 | 56 | 36 | 62 | 38 |
| 10 | 51 | 33 | 62 | 37 | 66 | 46 |
| 15 | 58 | 35 | 56 | 45 | 68 | 63 |
| 20 | 51 | | 65 | | 73 | |
| 30 | 52 | | 62 | | 75 | |
| 50 | 49 | | 63 | | 68 | |
| 100 | 14 | 15 | 37 | 8 | 38 | 20 |

[a]Glucosyltransferase preparations A and C are ammonium sulfate- and ethylamine-enzyme preparations, respectively. The guanidine-enzyme preparation was inhibited to the same extent as the ammonium sulfate-enzyme.

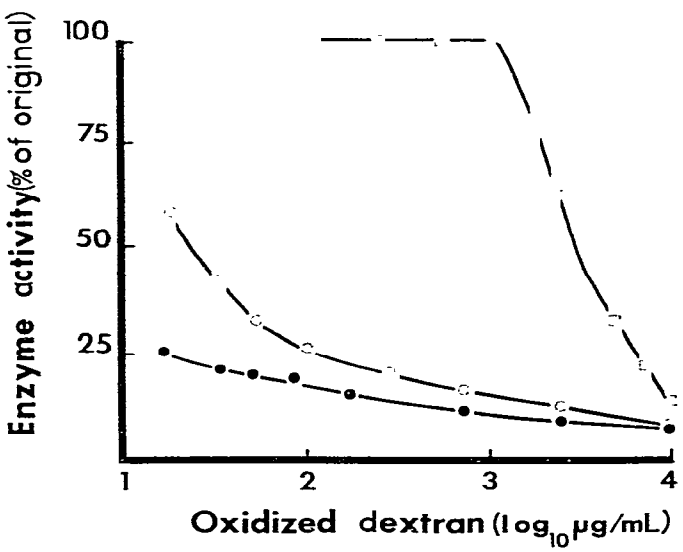


Fig. 4. Inhibition of glucosyltransferase pre-incubated with fully oxidized, clinical dextran. [Ammonium sulfate-enzyme (0.6 unit/mL) was pre-incubated for 20 min with the indicated concentrations of dextran (abscissa) that had been 10 (●), 100 (○), and 200% (□) oxidized. Substrate solution was then added, and glucan synthesis was determined after 30 min. Pre-incubation conditions and the assay of enzyme activity after pre-incubation were as described in Materials and Methods.]

TABLE II

INHIBITION OF GLUCOSYLTRANSFERASE BY NON-DEXTRAN COMPOUNDS

| *Inhibitor* | *Activity (% of the original) of glucosyltransferase after preincubation with different concentrations of non-specific inhibitors* | | |
|---|---|---|---|
| | *5 mg/mL* | *1 mg/mL* | *0.1 mg/mL* |
| Formaldehyde | 32 | 65 | 99 |
| Pyridoxal | 43 | 67 | 85 |
| Pyridoxine | 46 | 69 | 92 |
| Pyridoxamine | 64 | 62 | 108 |
| Pyridine | 51 | 73 | 104 |
| Nicotinic acid | 81 | 96 | 107 |
| Nicotinamide | 85 | 91 | 99 |
| D-Glucuronic acid | 73[a] | — | — |
| D-Gluconic acid | 135 | 120 | 107 |
| Fully oxidized amylose | 30 | 70 | 90 |
| Fully oxidized dextran | 33 | 80 | 100 |
| 10%-Oxidized dextran | 24 | 29 | 43 |

[a]Concentration of D-glucuronic acid was 10 mg/mL.

TABLE III

EFFECT OF PARTIALLY OXIDIZED ISOMALTODEXTRINS ON GLUCOSYLTRANSFERASE ACTIVITY

| *Degree of polymerization of isomaltodextrins*[a] | *Amount of periodate used in oxidation* | *Glucosyltransferase activity after preincubation with different amounts of isomaltodextrins (% of activity of enzyme preincubated alone)* | | |
|---|---|---|---|---|
| | | *20 μg/mL* | *1 mg/mL* | *10 mg/mL* |
| | 0 | 160 | 115 | 80 |
| 2–3 | equimolar | 90 | 100 | 85 |
| | large excess | 105 | 100 | 36 |
| | 0 | 165 | 105 | 70 |
| 3–5 | equimolar | 105 | 95 | 55 |
| | large excess | 120 | 100 | 30 |
| | 0 | 135 | 85 | 80 |
| 5–10 | equimolar | 75 | 90 | 75 |
| | large excess | 85 | 82 | 55 |
| 10 and higher | equimolar | 107 | 72 | 45 |
| | large excess | 105 | 105 | 52 |

[a]Isomaltodextrins were obtained by the acetolysis of clinical dextran.

tions of this polyaldehyde inhibited the enzyme (see Fig. 4) to about the same extent as oxidized amylose (see Table II).

10%-Oxidized derivatives of 9 dextran fractions, ranging in molecular weight from $10^4$ to $2 \times 10^6$, all inhibited the synthesis of D-glucan by glucosyltransferase.

The larger-size fractions (17 μg/mL) inhibited synthesis by 60–70%, compared to ~40% inhibition by the same concentration of the two smallest fractions ($10^4$ and $2 \times 10^4$). Ammonium sulfate- and guanidine-enzyme preparations were equally susceptible to inhibition. Clinical dextran incubated with sucrose and glucosyltransferase was modified by incorporation of D-glucose until its molecular weight had increased by 60%. Although, the modified D-glucan was ~150% more active as an acceptor molecule in the glucosyltransferase reaction, the 10%-oxidized derivatives of the original and modified dextrans did not differ in their ability to inhibit the synthesis of D-glucan when pre-incubated with enzyme.

*Inhibition of glucosyltransferase by oxidized oligosaccharides.* — Mixtures of oligosaccharides from the acetolyzate of clinical dextran, possessing d.p. 2–3, 3–5, 5–10, and 10 and higher, were partially oxidized with an equimolar proportion of periodate, or were fully oxidized with an excess of the oxidant. The partially oxidized, (1→6)-α-linked isomaltosaccharides caused a low inhibition, similar to the non-specific inhibition of fully oxidized oligosaccharides (see Table III). It is not yet known why unoxidized oligosaccharides activated the synthesis of D-glucan at a low concentration (20 μg/mL). Activation did not occur at higher concentrations (1 mg/mL and 10 mg/mL). Oligosaccharides from the acid hydrolyzate of dextran gave similar results (not shown).

Oligosaccharides (d.p. 3–8) from the acid hydrolyzate of *A. niger* α-glucan were oxidized with two molar proportions of periodate. Neither the oxidized nor the unoxidized (1→3)-α-linked oligosaccharides (1.4 mg/mL) inhibited the synthesis of D-glucan by glucosyltransferase under conditions in which preincubation of the enzyme with 10%-oxidized dextran (100 μg/mL) resulted in 65% inhibition. A mixture of (1→4)-α-linked maltosaccharides (d.p. 2–8), oxidized with one molar proportion of periodate, also did not significantly inhibit the synthesis of D-glucan.

The nonreducing trisaccharide lactulosucrose (β-$4^F$-galactosylsucrose) is a D-glucosyl donor for α-glucan synthesis in the reaction catalyzed by a glucosyltransferase of *Leuconostoc mesenteroides*[21]; this suggested that oxidation of the D-galactosyl group of the trisaccharide might afford a highly inhibitory, dialdehyde group, without decreasing the affinity of the enzyme for the intact, sucrose moiety. We were unable to detect synthesis of dextran from purified lactulosucrose by *S. mutans* 6715 glucosyltransferase; therefore, the oxidized derivative of this trisaccharide was not tested for inhibitory action. Instead, oxidized derivatives of the more-readily available trisaccharides raffinose and melezitose were tested. Like lactulosucrose, both contain a sucrose moiety, and are not substrates for the *S. mutans* enzyme.

Preincubation of glucosyltransferase with the oxidized derivatives (equimolar periodate) of raffinose (2 mg/mL) and melezitose (10 mg/mL) inhibited α-glucan synthesis by ~50%. The same concentrations of the unoxidized molecules had no effect. The two trisaccharide derivatives were better inhibitors than similarly oxidized maltotriose (50 mg/mL for 50% inhibition), but were several orders less potent as inhibitors than 10%-oxidized, clinical dextran (50 μg/mL for 50% inhibition). Sucrose treated with two molar proportions of periodate was not an inhibitor, but

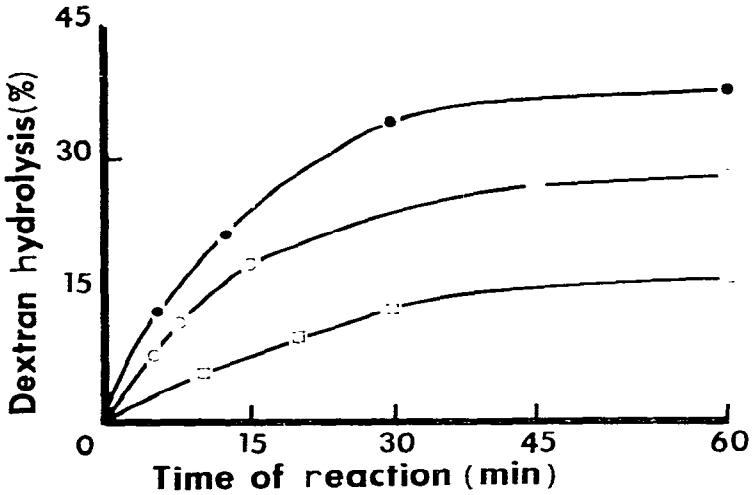


Fig. 5. Hydrolysis of clinical dextran and periodate-oxidized dextrans by dextranase. [Dextranase (2 units/mL) was incubated at 37° with clinical dextran (●), 10%-oxidized dextran (○), and 30%-oxidized dextran (□). The reaction mixtures contained 18 mg of polysaccharide/mL and 20mM sodium acetate buffer solution, pH 7.0. Samples were removed at the times indicated, and the extent of polysaccharide hydrolysis was determined from the release of copper-reducing sugars.]

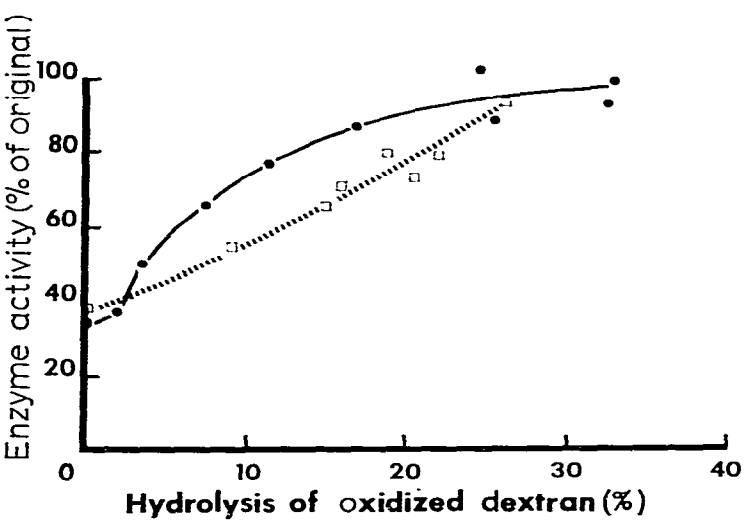


Fig. 6. Effect, on glucosyltransferase activity, of pre-incubation with partially oxidized dextrans subjected to different extents of hydrolysis by dextranase. [Glucosyltransferase (0.3 unit/mL) was preincubated for 20 min with 10%- (●) and 30%- (□) oxidized, clinical dextran (90 μg/mL) that had previously been hydrolyzed by dextranase (see Fig. 5) to the extents indicated. Pre-incubation conditions and assay of enzyme activity after pre-incubation were as described in Materials and Methods.]

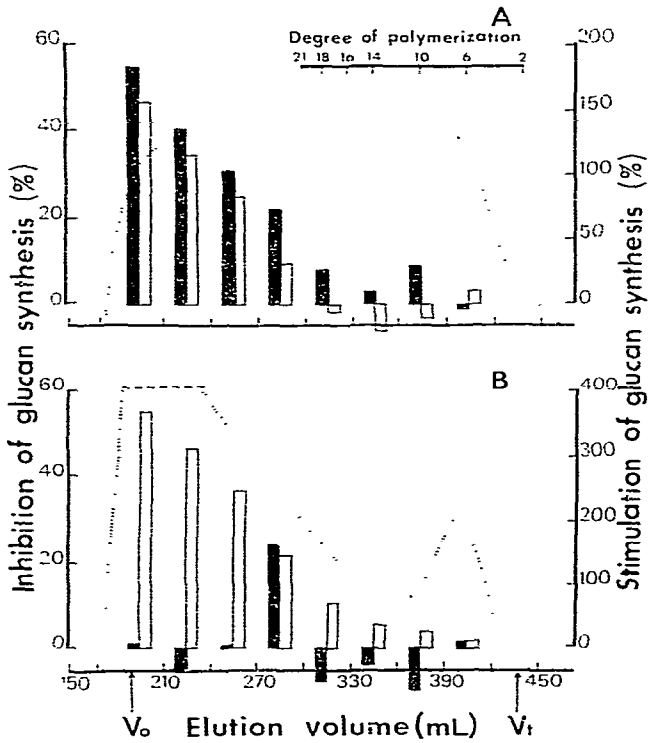


Fig. 7. Effects of components of the partial hydrolyzates of oxidized and unoxidized dextran on the synthesis of α-glucan by *S. mutans* glucosyltransferase. [Dextranase hydrolyzates (6%) of 20%-oxidized (A) and unoxidized (B) clinical dextran (100 mg) were applied in 50mM sodium phosphate–0.02% sodium azide solution, pH 7.5 (1.0 mL) to a Bio-Gel P6 column (2.6 × 92 cm). The column was irrigated at a flow rate of 5 mL/h with the same phosphate solution, and 3- to 5-mL fractions were collected at room temperature. The relative concentrations of carbohydrate in the fractions were determined by the phenol–sulfuric acid method (dotted line). Fractions in 30-mL increments of the elution volume (210–240, 240–270 mL, etc.) were combined, lyophilized, and dissolved in a small volume of phosphate solution. Carbohydrate from each solution was pre-incubated (125 μg/mL) with enzyme in order to determine its relative ability to inhibit glucan synthesis by enzyme action in the presence of dextran acceptor (solid bars), and the same amount was directly incubated (83 μg/mL) with enzyme and sucrose, in order to determine its relative ability to stimulate α-glucan synthesis by enzyme action in the absence of dextran acceptor (open bars). Condition for the assay of α-glucan synthesis and its inhibition were as described in Materials and Methods.]

sucrose treated with one molar proportion thereof caused a 50% decrease in the synthesis of D-glucan when it was preincubated with glucosyltransferase. However, unoxidized sucrose caused a similar inhibition, indicating that interaction of dialdehyde groups with the enzyme was not responsible. A progressive loss of the activity of glucosyltransferase incubated with sucrose has been reported[16].

*Inhibition of glucosyltransferase by products from the hydrolysis of oxidized dextran.* — Endo-dextranase hydrolyzed clinical dextran at a higher rate, and to a greater extent, than it hydrolyzed 10%- and 30%-oxidized dextrans (see Fig. 5). Thin-layer chromatograms showed that isomaltose was the major, final product in

the three reaction-mixtures, but the oxidized dextrans yielded greater amounts of enzyme-resistant oligosaccharides, which were not characterized. Partial-hydrolysis products of the oxidized dextrans were pre-incubated with glucosyltransferase under standard conditions, and their effect on the enzymic synthesis of α-glucan was determined.

The 10%- and 30%-oxidized derivatives at low concentration (90 μg/mL) exhibited more than half their original potencies as inhibitors after 10% hydrolysis, but lost all inhibitory action against glucan synthesis after 25% hydrolysis (see Fig. 6). Higher concentrations of the oxidized-dextran hydrolyzates (0.9 and 9 mg/mL) gave similar results, but glucan synthesis was not affected by pre-incubation of the enzyme with any of the partial hydrolyzates of unoxidized, clinical dextran.

Separations, on a column of Bio-Gel P-6, of the components of enzymic hydrolyzates (6% hydrolysis) of unoxidized and 20%-oxidized dextran are shown in Fig. 7. Different fractions from the elution profiles of the unoxidized- and oxidized-dextran hydrolyzates were preincubated with glucosyltransferase in order to determine their relative abilities to inhibit the synthesis of α-glucan by the enzyme acting on sucrose in the presence of acceptor dextran (1 mg/mL). Fractions from the hydrolyzates were also incubated directly with glucosyltransferase and sucrose, in order to determine their abilities to stimulate α-glucan synthesis in the absence of acceptor dextran. Both the inhibitory and the stimulatory capacities of the oxidized-dextran fractions increased with molecular size, and became significant in fractions whose elution volumes corresponded to degrees of polymerization of >16–20 D-glucosyl units (see Fig. 7A). The same pattern was evident for the two-fold greater stimulatory capacity of corresponding fractions of the unoxidized-dextran hydrolyzate but, with the exception of one fraction, they did not exert a significant inhibitory effect when pre-incubated with glucosyltransferase (see Fig. 7B). Inhibition by the single fraction in the clinical-dextran hydrolyzate was not confirmed in other experiments, in which clinical dextran was subjected to different degrees of oxidation and hydrolysis, but otherwise essentially similar results were obtained. Similar separations on a calibrated, Sephadex G-50 column indicated that a d.p. ~20 D-glucosyl units was required for inhibitor/acceptor molecules.

*Inhibition of glucosyltransferase by other compounds.* — A number of non-dextran compounds containing aldehydic functional groups were tested as inhibitors of glucan synthesis by glucosyltransferase. Completely oxidized amylose was a poor enzyme-inhibitor, comparable in potency to completely oxidized dextran (see Table III). Other nonspecific inhibitors containing an aldehyde group were formaldehyde and pyridoxal, but pyridoxine and pyridoxamine, neither of which contains an aldehyde group, were equally as inhibitory as the aromatic aldehyde (see Table II). Acarbose (BAYg 5421), a specific inhibitor of alpha amylase and α-D-glucosidase, did not inhibit glucosyltransferase when preincubated (1 mg/mL) with enzyme, but it did inhibit glucan synthesis (62%) when it was included at high concentration (4.6 mg/mL) in the enzyme reaction-mixture. However, in no case was the inhibition of *S. mutans* glucosyltransferase by the nonspecific compounds comparable in potency

to the specific inhibition that occurred when the enzyme was preincubated with partially oxidized dextrans.

## DISCUSSION

Our search for potent inhibitors of *S. mutans* glucosyltransferase was conducted with impure enzyme-preparations for convenience and in the belief that inhibitors that act efficiently only on pure enzyme are unlikely to be potent inhibitors *in vivo*. However, adequate controls were included in all studies, because crude enzyme-preparations may contain significant levels of dextranase[22] and protease[23] which, if uncontrolled, would lead to "anomalous" results in the inhibition assays. Enzyme solutions may also contain α-D-glucan formed from small amounts of sucrose in the components of the *S. mutans* growth-medium[24]. Because addition of clinical dextran to enzyme solutions increases glucan synthesis, and decreases enzyme inhibition by 10%-oxidized dextran (see Fig. 2), the presence of different proportions of endogenous α-D-glucan in glucosyltransferase preparations may similarly affect enzyme action. Results obtained with crude enzyme-preparations must, therefore, be interpreted with caution.

The exact point of oxidation of dextran by periodate is not yet known, but preliminary results (data not shown), obtained by the Smith-degradation method[25], indicated that even high degrees of oxidation leave sequences of unmodified D-glucosyl units that retain affinity for the glucosyltransferase binding-site; this may explain why 100%-oxidized dextran inhibited some glucosyltransferase preparations (see Fig. 4), whereas complete oxidation (200%), in destroying all dextran-like structure, decreased enzyme inhibition to a level characteristic of oxidized derivatives of nonspecific polysaccharides, such as amylose (see Fig. 4 and Table II). 10%-Oxidized dextran fractions of different molecular size ($10^4$ to $2 \times 10^6$) apparently possess the affinity required for high inhibitory potency towards glucosyltransferase, but this inhibitory potency was not increased when, prior to partial oxidation, clinical dextran (mol. wt. 60,000–90,000) was converted into a superior acceptor-substrate for the glucosyltransferase reaction.

Oxidized oligosaccharides were tested because of the possibility that some might exhibit affinity for the enzyme and be potent inhibitors, capable of penetrating the matrix of insoluble dextran and dental plaque in which glucosyltransferase activity is found in *vivo*. In a random, partial oxidation, a proportion of the reducing oligosaccharides would be oxidized at internal D-glucosyl residues, and a proportion of the nonreducing trisaccharides raffinose and melezitose would retain an intact sucrose moiety. However, poor inhibition of soluble glucosyltransferase by mixtures of partially oxidized oligosaccharides (see Table III and text) made it unlikely that they contained a potent inhibitor that would affect enzyme activity associated with *S. mutans* cells or other plaque components. Our observation (not shown) that enzyme inhibitor was not formed from clinical dextran when mixtures of the dextran and oligosaccharides were treated with a 10% molar proportion of periodate (0.1

mol of oxidant/mol of hexose) suggests that preferential oxidation at the reducing end of oligosaccharides may be responsible for their lack of inhibitory potency.

As the dialdehyde structures required for enzyme inhibition probably remain intact in oxidized dextran fragmented by the *endo*-action of dextranase, the loss of inhibitory power that accompanies this fragmentation (see Fig. 6) indicates that the size of the molecule containing the inhibitory dialdehyde is critical for enzyme affinity. Similarly, hydrolysis of clinical dextran decreases[26] its affinity as an acceptor for glucan synthesis by the glucosyltransferase of *S. mutans* K-1R, and (1→6)-α-linked, oligosaccharide acceptors containing at least 8 D-glucosyl residues are required, in order to stimulate the synthetic reaction of *S. mutans* 6715 glucosyltransferase[27]. The partially purified enzyme-preparations used in our inhibitor studies required oligosaccharides containing at least 10 to 14 D-glucosyl units for stimulation of glucan synthesis (see Fig. 7B), and a slightly larger size apparently endowed oxidized oligosaccharides with the enzyme affinity required in order to enable them to inhibit glucan synthesis in the presence of acceptor dextran, and to stimulate glucan synthesis in the absence of alternative acceptor (see Fig. 7A).

Unoxidized, clinical dextran protected against (Fig. 3), but did not readily reverse[10], the inhibition of glucosyltransferase preincubated with oxidized dextran. This suggests that a relatively stable covalent bond is formed between dialdehyde groups in the oxidized inhibitor and a reactive group near the enzyme acceptor-site. However, glucan synthesis by the enzyme in the presence of oxidized dextran (see the text) or oxidized oligosaccharides (see Fig. 7A) was substantially greater than in the absence of an alternative acceptor, and it is concluded that the enzyme-bound, oxidized molecules retain some acceptor function; this is consistent with the failure of even high concentrations of oxidized dextran to inhibit all glucan synthesis (see Fig. 1).

There is a limit to which a given amount of clinical dextran can sustain synthesis of α-glucan by *S. mutans* glucosyltransferase[26], and, when enzyme-bound, dextran molecules reach this limit, they presumably dissociate from the enzyme to allow other molecules to bind. Glucan synthesis then proceeds until all available dextran molecules are modified to the extent that they no longer serve as efficient acceptors. Thus, the inhibition that results from pre-incubation of glucosyltransferase with oxidized dextran (see Fig. 1) may be attributed to the failure of the enzyme to dissociate readily from oxidized-dextran molecules. The bound enzyme is, therefore, unable to use the unoxidized dextran present in the reaction mixture as acceptor for continued α-glucan synthesis.

The different extents of inhibition of glucan synthesis catalyzed by different enzyme-preparations, and the disparity between the inhibition of glucan synthesis and reducing-sugar release (see Fig. 3), suggest that enzyme preparations contain catalytic activities that have different acceptor requirements. As the inhibitory action of oxidized dextran is directed only against α-glucan synthesis for which an acceptor of high molecular weight is required, α-glucan synthesis by other enzymic components in dental plaque would be unaffected by oxidized-saccharide inhibitors. The protective

action of endogenous α-glucans, and the steric factors engendered by the relatively large size required for potent inhibition, would further decrease the effectiveness of oxidized-saccharide inhibitors and, therefore, further detract from their potential value as therapeutic agents for the lessening of dental caries *in vivo*.

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