

## Effect of Salts on Water-Insoluble Glucan Formation by Glucosyltransferase of *Streptococcus mutans*

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Received for publication 11 October 1978

The formation of water-insoluble glucan by extracellular glucosyltransferase from *Streptococcus mutans* 6715 was found to be greatly stimulated by various mono- or divalent cations. An enzyme preparation, obtained by ethanol fractionation, was able to catalyze the formation of water-insoluble glucan from sucrose in the presence of monovalent cations above 100 mM or divalent cations above 20 mM at neutral pH. As the concentration of monovalent and divalent cations was reduced to below 10 mM and 1 mM, respectively, the formation of insoluble glucan decreased to a negligible amount. High concentrations of these cations were found to stimulate the formation of insoluble glucan in the following ways: (i) it increased the activity of total glucosyltransferase up to 1.6- and 2.7-fold in the absence and presence of a primer dextran, respectively, and (ii) it changed the formation of soluble glucan to insoluble. It was postulated that one of the essential factors for the formation of insoluble glucan would be to keep more than two water-soluble glucan chains close to enzyme aggregates and that such interaction could be enhanced by the presence of high cation concentrations.

The formation of water-insoluble glucan from sucrose is believed to be a major factor in accumulations of *Streptococcus mutans* on smooth surfaces (15, 22, 25, 33, 35, 39). The water insolubility of the product is generally thought to be due to its high content of  $\alpha$ -1,3 linkages in addition to  $\alpha$ -1,6 linkages (1, 2, 8, 17). The  $\alpha$ -1,3 and  $\alpha$ -1,6 linkages are both present at C3 and C6 atoms of the glycosyl residues as the branching points (8, 36) as well as at main chains. Since some water-soluble glucans were found to contain similar proportions of linkages as water-insoluble ones (36), the size or distribution of such linkages in a glucan molecule might determine its solubility. Based on these observations, the de novo synthesis of the water-insoluble glucan seems to require a cooperative interaction of a group of enzymes, each of which would produce  $\alpha$ -1,3,  $\alpha$ -1,6, and the branching linkages, although the possibility that one kind of enzyme is responsible for all these linkages cannot be excluded at the present moment. In *S. mutans* culture supernatant, the enzyme(s) responsible for water-insoluble glucan formation is actually present as a high-molecular-weight complex with a high content of carbohydrate (5, 10, 35, 38). These complexes produce glucans of variable water solubility and of variable linkage composition in the presence of soluble dextran and exogenous dextranase (8, 12, 37). For example, exogenous soluble dextran caused a decrease in the formation of water-insoluble glucan and an

increase in the water-soluble forms (23, 31); it also caused a decrease in molecular size of the enzyme complexes or aggregates (13, 23). Therefore, it is apparent that insoluble glucan formation can be affected by physical factors, as well as genetic and metabolic factors (6, 9, 20, 21, 24, 30).

In this paper we present evidence that salt concentration is one of the essential physical factors influencing insoluble glucan formation and that high concentrations of salts not only increase the velocity of glucan synthesis but also shift the glucan formed from water soluble to water insoluble.

### MATERIALS AND METHODS

**Cultural conditions.** Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) was fortified with salts (19) and 40 g of fructose, pretreated with 3 ml of a yeast invertase preparation (6.5  $\mu$ SI/ml at 20°C, Wako Pure Chemicals, Osaka) for 30 min at 30°C to increase the amount of the extracellular enzymes (38, 42), and then autoclaved. In the modified broth, *S. mutans* strain 6715 (from H. D. Slade, Northwestern University Medical and Dental School, Chicago, Ill.) was grown at 37°C for 17 h. At the end of the growth phase, the turbidity and the pH of the culture were 3.52 at 660 nm and 5.07, respectively.

**Enzyme preparation.** The supernatant of the culture (2 liters) was obtained by centrifugation and adjusted to pH 4.5 with dilute phosphoric acid. While the temperature of the supernatant was kept below 0°C, 800 ml of cold absolute ethanol was added to a final concentration of 28% (vol/vol). After 2 h at 4°C,

the precipitate formed was collected by centrifugation and suspended in 100 ml of distilled water. The enzyme preparation contained 2.98 mg of protein and 0.24 mg of polysaccharide per ml and was kept at 4°C in the presence of 0.01% Merthiolate. The same preparation was used throughout the entire course of this study. The average water-insoluble glucan formation activity during 15 h of incubation at 37°C was 37.0 and 12.8  $\mu\text{mol}$  of glucose per ml per min in the presence and absence of *Leuconostoc mesenteroides* dextran T10 (Pharmacia Fine Chemicals, Piscataway, N.J.), respectively. The recovery of the total glucosyltransferase activity from the culture supernatant was 82%. The electrolyte concentration of the preparation was 1.52 mM, when measured by a CM-30 conductivity meter (Shimazu, Kyoto) using KCl as a standard. Since this preparation was diluted 175 times with buffer during the measurement of enzyme activity, any effects of electrolytes in the preparation would be negligible. The total fructosyltransferase activity was 0.4  $\mu\text{mol}$  of fructose per ml per min, measured by counting [ $^3\text{H}$ ] fructose incorporated into polysaccharide from [1-*fructosyl*- $^3\text{H}$ ]sucrose (New England Nuclear Corp., Boston, Mass.). This enzyme activity was disregarded in the present study because it was only 3% of the total glucosyltransferase activity. Frequent analysis of the enzyme revealed that almost all of the glucosyltransferase activity was found in the precipitate. Therefore, the preparation was gently swirled so that it became smooth and translucent for pipetting. The precipitate was perfectly suspended in the reaction mixtures used in the present study. The enzymatic activities of the preparation did not change significantly after 6 months of storage.

**Enzyme assays.** Sodium acetate (pH 4.5, 5.0, and 5.5) and sodium phosphate buffers with a final concentration of 5 mM were used. Unless otherwise specified, phosphate buffer at pH 6.5 was used. In studies on effects of divalent and trivalent cations, sodium acetate buffer (pH 5.0) was used to minimize pH changes and to prevent precipitation of salts. The pH changes of buffers after addition of salts were within  $\pm 0.2$ . Since precipitate formation with 5 mM  $\text{FeCl}_3$  and  $\text{Al}_2(\text{SO}_4)_3$  was significant, studies using high concentrations of these trivalent cations could not be carried out.

Reaction mixtures were composed of 20  $\mu\text{l}$  of the enzyme preparation described above, 0.01% Merthiolate, 50 mg of sucrose, and various concentrations of salts, with and without 1.2 mg of exogenous dextran T10 in 5 mM buffer in a total volume of 3.5 ml. Incubation was carried out in a reaction tube at 37°C for 15 h, and the reaction was terminated by placing the tube in a boiling water bath for 5 min.

The water-insoluble glucan formed was sedimented at 2,000  $\times g$  for 10 min and washed three times, each with 5 ml of distilled water. The sediment was smoothly suspended in 1 ml of distilled water by agitation, and a 0.1-ml sample of the sediment was assayed at 490 nm by the phenol-sulfuric acid method (7), using glucose as a standard.

For the analysis of water-soluble glucan, 1 ml of the supernatant was precipitated with four volumes of absolute ethanol. The precipitate was washed three times with 5 ml of 75% ethanol, dissolved in 1 ml of

distilled water, and assayed by the phenol-sulfuric acid method. Care was taken to adjust the salt concentrations of solutions to about 0.1 to 0.2 M by adding NaCl before addition of ethanol, since sedimentation of dextran T10, T2000, and soluble dextran formed by the enzyme preparation was found to be incomplete at lower salt concentrations. When dextran T10 was added to reaction mixtures, the water-soluble glucan measured by this method included the exogenous dextran as well as soluble dextran formed.

The synthesis of total glucan was measured by the incorporation of [ $^{14}\text{C}$ ]glucose into glucan. To the reaction mixture described above, [*U*-glucosyl- $^{14}\text{C}$ ]sucrose (New England Nuclear Corp.) was added to give  $7.4 \times 10^4$  dpm per 50 mg of total sucrose in the reaction tube. After incubation, the material was precipitated with ethanol and washed as described above. The glucan synthesized was directly solubilized with 0.2 ml of 50% nitric acid at 100°C for 30 min. Without being neutralized, it was transferred into a scintillation vial containing 10 ml of scintillation fluid, which consisted of 700 ml of toluene, 300 ml of ethanol, 4 g of 2,5-diphenyloxazole, and 0.1 g of 1,4-bis-2-(5-phenyloxazolyl)benzene. Samples were counted with an Aloka liquid scintillation system LSC-673 (Aloka Co. LTD, Tokyo). This system was equipped with an internal  $\gamma$ -ray standard, and a value of counts per minute was automatically transformed into disintegrations per minute, compensating for the effects of quenching. Although the fluid containing the sample was highly acidic and of high quenching, the solution was perfectly clear when containing several milligrams of water-insoluble glucan, and the amount of glucose incorporated could be quantitatively determined within 4% error.

Total reducing sugars were measured at 500 nm by the method of Somogyi (41), using glucose as a standard.

Fructose and glucose formed were individually determined by using a glucose/fructose UV-test kit (Boehringer Mannheim GmbH, Mannheim). A 0.1-ml sample of the supernatant of the reaction mixture was directly used.

The controls, which contained the enzyme preparation pretreated in boiling water for 5 min, were run to correct for nonenzymatic activities in all the enzyme assays described here. Based on the net amounts of sugar products determined, water-insoluble and water-soluble glucan formation activities, total glucan synthesis activity, and invertase activity were calculated and expressed as milligrams of glucan or sugar formed during 15 h of incubation at 37°C in a reaction tube containing 3.5 ml of reaction mixture, for ease of quantitative comparison. The analytical methods employed here were not necessarily the most sensitive, but they were quantitatively reliable within at least 5% error.

**Other assays.** Protein and carbohydrate contents of the enzyme preparation were determined by the method of Lowry et al. (27) using serum albumin as a standard and by the phenol-sulfuric acid method (7) using glucose as a standard. A qualitative analysis of reducing sugars were performed by thin-layer chromatography as reported (29), with the following modifications. The supernatant obtained by centrifugation

after incubation was desalted through a mixed bed of Dowex 50W-X4, 1-X4 (Dow Chemicals, Midland), and after lyophilization the sample was chromatographed in *n*-butyl alcohol-pyridine-water (4:6:3) on Silica Gel 60F-254 (E. Merck, Darmstadt) and then stained with the alkaline silver nitrate.

## RESULTS

**Effect of salt concentration on water-insoluble glucan formation.** The formation of water-insoluble glucan from sucrose by the ethanol-precipitated enzyme preparation derived from *S. mutans* 6715 culture supernatant was greatly stimulated by the addition of NaCl to reaction mixtures containing low concentrations (5 mM) of buffer (Fig. 1). In the absence of primer dextran (Fig. 1A), insoluble glucan formation was negligible if the concentration of NaCl was below 10 mM at pH 4.5 to 7.5 and more than 100 mM NaCl was required for maximum activation. In the presence of an exogenous dextran T10 (Fig. 1B), NaCl above 100 mM was also required at pH 6.5 and 7.5 for optimal enhancement, which was about 2.5-fold the non-primed activity. At the lower pH's of 5.5 and 4.5, 200 mM or more NaCl was required, and at the higher pH of 8.5 50 mM NaCl was the optimal concentration.

To determine whether the extent of stimulation varied with the nature of the cation or anion, various salts were added to the reaction mixtures at pH 5.0 (Fig. 2). Other salts such as KI, KCl, KClO<sub>4</sub>, K<sub>2</sub>SO<sub>4</sub>, NH<sub>4</sub>NO<sub>3</sub>, and Na<sub>2</sub>SO<sub>4</sub> were also used in this study, but the results are not shown in the figure. The optimal concentration of monovalent cations was 400 mM at this pH, whereas the maximal stimulation by divalent cations occurred in the vicinity of 80 mM. Much higher concentrations of cations were in-

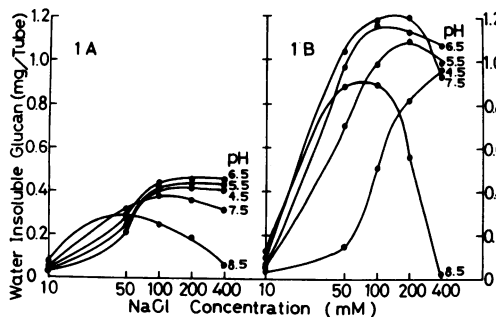


FIG. 1. Effect of NaCl concentration on water-insoluble glucan formation at various pH's. NaCl was added to each reaction mixture with (B) or without (A) 1.2 mg of dextran T10 in 3.5 ml of 5 mM buffer. After 15 h of incubation at 37°C, the water-insoluble glucan formed was measured by the phenol-sulfuric acid method.

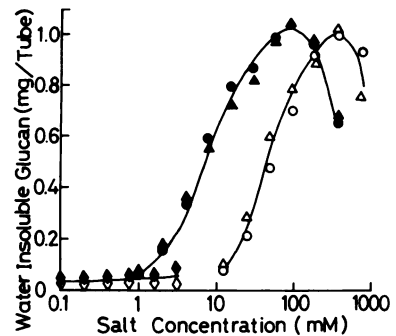


FIG. 2. Effect of various salts on water-insoluble glucan formation. The buffer used was 5 mM sodium acetate buffer (pH 5.0) in the presence of 1.2 mg of dextran T10. The salt concentrations shown are with regard to the cation components. Symbols: ○, NaCl; △, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; ●, CaCl<sub>2</sub>; ▲, BaCl<sub>2</sub>; ◇, FeCl<sub>3</sub>; ◆, Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>.

hibitory to insoluble glucan formation. Addition of trivalent cations of Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub> and FeCl<sub>3</sub> up to 3.2 mM did not stimulate this reaction, and higher concentrations of these ions could not be added because of heavy precipitate formation in the buffer system at this pH. The fact that the extent of stimulation depended on the concentration of cations would indicate that the concentration and the nature of anions such as I<sup>-</sup>, NO<sub>3</sub><sup>-</sup>, Cl<sup>-</sup>, SO<sub>4</sub><sup>2-</sup>, and ClO<sub>4</sub><sup>-</sup> were not the determinant components of the stimulation.

**Effect of salt concentration on other enzymatic activities.** Since water-insoluble glucan formation was greatly stimulated by salt, it was of interest to know what products were formed in low salt concentration instead of insoluble glucans, and to investigate other enzymatic activities that might be influenced by salt concentration. To standard reaction mixtures of 5 mM sodium phosphate buffer (pH 6.5) various concentrations of NaCl were added, and water-insoluble and -soluble glucans and reducing sugars formed were measured (Fig. 3). Throughout this experiment, it was noticed that the amount of total reducing sugars formed was almost equal to the sum of free fructose and glucose. This was supported by the result that the amounts of other reducing sugars, such as isomaltose, were negligible when analyzed by thin-layer chromatography (data not shown). The amount of total glucan formed also corresponded to the amount of free fructose less that of free glucose. These data indicate that quantitatively significant amounts of invertase and glucosyltransferase were present in the enzyme preparation.

The invertase activity, which was estimated by the amount of free glucose, was about 0.3 to

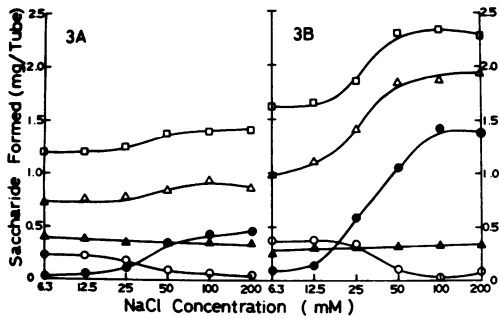


FIG. 3. Effect of NaCl concentration on various saccharide formation activities. Standard reaction conditions were used in sodium phosphate buffer (pH 6.5) in the presence (B) or absence (A) of 1.2 mg of dextran T10, with varying concentrations of NaCl. Symbols: ●, water-insoluble glucan; ○, water-soluble glucan; ▲, free glucose formed; △, free fructose formed; □, total reducing sugars.

0.4 mg of glucose in 15 h per tube, and was affected neither by salt concentration nor by a primer dextran (Fig. 3A and B). The activity was comparable to that of total glucan formation (0.25 to 0.5 mg of glucan) in the absence of primer (Fig. 3A) and about 50 to 20% of the activity of glucan formation in the presence of primer (Fig. 3B).

The main glucan product formed at low salt concentrations (below 1.2 mM) was water soluble instead of water insoluble (Fig. 3A and B). With increasing concentrations of salt, the formation of soluble glucan decreased to almost zero at 100 mM and was accompanied by an increase of water-insoluble glucan. However, the decrease in soluble glucan accounted for only about 30 and 50% of the amount of increase in insoluble glucan in the absence and presence of a primer dextran, respectively. The remaining 70 and 50% of the insoluble glucan could be explained by a stimulation of glucosyltransferase activity (that is, total glucan formation activity) in the absence and presence of dextran (Fig. 3A and B). The glucosyltransferase was stimulated by high salt up to 1.6- and 2.7-fold in the absence and presence of the primer.

**Effect of salt pulse on water-insoluble glucan formation.** The rate of water-insoluble glucan formation has been reported to be constant during the first hour of incubation following a variable initial lag period (5, 32). In the present study, insoluble glucan formation in reaction mixtures of low enzymatic activity was almost linear for 15 h, with about a 2-h initial lag, in the presence of primer and high salt concentrations (data not shown). A similar curve was reported previously (33). At low salt concentrations, soluble glucan formation was also linear

for 15 h without a time lag (not shown). To see if the soluble glucan formed at the initial stages of incubation in low salt concentrations could be incorporated into insoluble glucan when pulsed with NaCl, and, if so, to measure the additional incubation time required for maximum insoluble glucan formation, NaCl was added to low-salt reaction mixtures at various times after incubation, and the incubation was continued in high salt for the remainder of the total 15 h (Fig. 4A and B).

In the absence of a primer dextran (Fig. 4A), soluble glucan that accumulated during the initial incubation up to 6 h seemed to be effectively incorporated into insoluble glucan during subsequent incubation in a high salt concentration, since the maximal amount of insoluble glucan was formed. However, when the NaCl pulse was delayed, a significant amount of soluble glucan remained in the reaction mixture, and the amount of insoluble glucan was almost proportional to the latter incubation time. In the presence of a primer dextran (Fig. 4B), the pulse of NaCl could wait for about 3 h for maximal formation of insoluble glucan. It was noticed that, in the presence of more than a certain amount of soluble glucan, the velocity of insoluble glucan formation was constant in high salt concentrations (Fig. 4B).

**Priming effect by exogenous dextran in low and high concentrations of salt.** Only the soluble glucan-forming activity was stimulated by the dextran primer at low salt concentrations, since the increase in total reducing sugars was close to that in the soluble one (Fig. 5A).

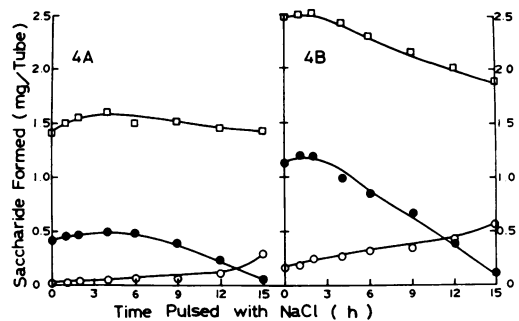


FIG. 4. NaCl pulse and the formation of glucans and reducing sugars. Standard reaction mixtures without added salt were first incubated in the presence (B) and absence (A) of dextran T10 for various time intervals, and then NaCl was added to make a final concentration of 100 mM. The incubation was continued for up to a total time of 15 h. Symbols: ●, water-insoluble glucan; ○, water-soluble glucan; □, total reducing sugars.

At high salt concentrations, insoluble glucan-forming activity was stimulated about fourfold by 1.2 to 3.2 mg of dextran T10 in 3.5 ml of reaction mixture. The amount of increase of the priming effect corresponded to that of total glucan formation and also to that of total reducing sugars (Fig. 5B), indicating that the activity induced by the primer dextran was only that of the insoluble glucan. Higher concentrations of the primer, however, were inhibitory to the formation of insoluble glucan, and soluble glucan was preferentially formed.

### DISCUSSION

It was found in the present study that high concentrations of salt stimulated glucosyltransferase activity up to 2.7-fold and also shifted the product from water soluble to insoluble. Non-specific salt-dependent activations of enzymes have been reported in several papers (26, 40, 45). Optimal concentrations of monovalent (0.1 M) and divalent (0.05 M) salts activated dihydrofolate reductase from 1.5- to 1.7-fold (45), and salts such as KCl and NaCl (0.125 M) stimulated a polynucleotide kinase up to sixfold (26). However, to our knowledge, changes in water solubility of the glucosyltransferase reaction product by salt concentrations have not yet been reported.

The enzyme preparation used in this study consisted of high-molecular-weight aggregates that contained most of the extracellular glucosyltransferase and possibly some other enzymes such as fructosyltransferase and dextranase (3, 5, 12, 43). However, only the glucosyltransferase and invertase-like activities were significant under the assay conditions employed. Since puri-

fied glucosyltransferase from *S. mutans* HS6 (10) and 6715 (5) and *L. mesenteroides* (16) consistently formed a considerable amount of free glucose from sucrose, the invertase-like activity of this preparation might be due to the glucosyltransferase itself.

When aggregates obtained by precipitation or ultrafiltration were subjected to further purification procedures, insoluble glucan-forming activity was reported to decrease far more rapidly than the increase in the degree of purification (5, 23, 35). The insoluble glucan could increase to a great extent in the presence of the glucosyltransferase-synthesizing  $\alpha$ -1,6 glucan and exogenous dextran (5, 12, 37). These observations indicate that not only the enzyme of  $\alpha$ -1,3 glucan but also other enzymes and polysaccharides contained in the aggregates are important for insoluble glucan formation.

Exogenous dextran has been reported to inhibit insoluble glucan formation (5, 14, 20) and to stimulate soluble glucan synthesis (10, 11, 28). More recent studies report that the addition of increasing amounts of dextran shifts the product from water insoluble to soluble (31, 32, 37). In the present study it was found that whether dextran stimulates insoluble glucan formation depends on salt concentration (Fig. 3), amount of dextran (Fig. 4), and incubation time (Fig. 5). As indicated by the present standard conditions, relatively lower concentrations of dextran and longer incubation times favor the formation of insoluble products. Results of the present study also suggest that  $\alpha$ -1,3 glucan synthesized by *S. mutans* could remain soluble under certain conditions.

It would be appropriate to divide the effects of salt on the formation of water-insoluble glucan into two factors: that is, stimulation of total glucosyltransferase activity and the effects on product solubility.

As to the former effect, the enzyme was found to require a certain concentration of monovalent or divalent salts in addition to a primer for maximal catalytic activity, as has been seen with some other enzymes (26, 45). As previously reported (10, 34), pH also critically affects the activity. Different activities observed at various pH values (Fig. 1) could be interpreted to be mainly due to total catalytic activity, since the total reducing sugars formed were always parallel to the amount of insoluble glucan at higher concentrations of salt (data not shown). Another stimulation factor recently reported is phosphoglycerides, which stimulate the enzyme by binding to a site different from either the glucosyl donor or glucosyl acceptor (primer) binding sites (18). More than 1 to 10 mM  $\text{Ag}^+$ ,  $\text{Pb}^{2+}$ , or  $\text{Hg}^{2+}$  almost completely inhibited the total

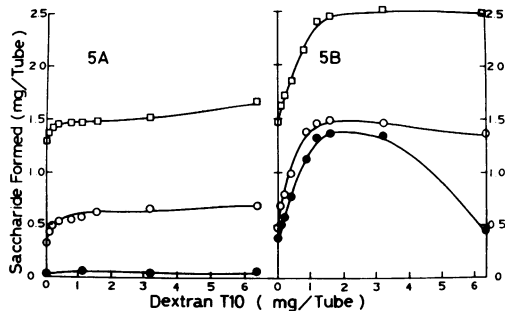


FIG. 5. Priming the formation of glucans and reducing sugars by exogenous dextran T10 in low (A) and high (B) concentrations of salt. Standard assay conditions were used with 12.5 mM NaCl (A) or with 100 mM NaCl (B). Symbols:  $\square$ , total reducing sugars;  $\circ$ , total glucan formed, estimated by measuring  $^{14}\text{C}$ -labeled glucose incorporated into glucan;  $\bullet$ , water-insoluble glucan formed, measured by the phenol-sulfuric acid method.

catalytic activity at pH 5.0 (data not shown), as has been reported (4).

As to the effect of salt on product solubility, this does not depend solely on total ionic strength or conductivity as measured by a conductometric meter; it also seems to be independent of the valence or the concentration of anion components. It does seem to depend, however, on the concentration of cations, as already mentioned. It is possible that the glucan formed might be slightly negative charged. The glucosyltransferase is also negative charged at a near neutral pH, as has been shown in most reports concerning purification or properties of this enzyme. Therefore, increasing concentrations of cations would be able to decrease the electrical repulsion energy among the negative-charged glucans and enzyme aggregates. It is also reasonable to consider that the insoluble glucan may be a three-dimensional covalent-bond structure, based on the following observations: (i) insoluble glucan, once formed, remains insoluble despite several washes with distilled water; (ii) it could not be solubilized by most solvents; (iii) the major part of the alkaline-solubilized glucan could be reprecipitated after neutralization (36); and (iv) the product contained a significant amount of branching linkages in addition to  $\alpha$ -1,3 and  $\alpha$ -1,6 linkages (8, 17, 36). It could be further postulated that, in high salt concentrations, glucan chains would be close to enzyme aggregates so that the enzyme could bind the chains covalently, and that, in low salt concentrations, the possibility of covalent binding decreases to a point where the product remains water soluble. An enhanced affinity between sweet-corn (1,4)- $\alpha$ -D-glucan synthetase and the polysaccharide primer by high salt concentration has been reported (40). This might also partially support the above postulation.

Our present findings indicate that the formation of insoluble glucan requires specific physical or structural conditions of the product and the enzyme in addition to optimal catalytic activity. These special requirements might be seen in the formation of other insoluble polymers, but they have not yet been reported. Further studies on the physical factors regulating insoluble glucan formation by *S. mutans* might provide clues leading to effective prevention of smooth-surface dental caries.

#### ACKNOWLEDGMENT

We thank C. Lee (Northwestern University Medical School, Chicago, Ill.) for critical reading of the manuscript.

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