JOURNAL OF BACTERIOLOGY, Oct. 1974, p. 287-294
Vol. 120, No. 1

Streptococcus mutans Dextransucrase: Requirement for Primer Dextran

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Received for publication 17 June 1974

Dextran stimulation (priming) of the dextransucrase (EC 2.4.1.5) from *Streptococcus mutans* strain 6715 was studied. The dextransucrase activity in supernatant fluids from glucose-grown cultures was shown to be partially primer dependent. During extended storage at 4 C the enzyme retained its activity. However, the ability to make dextran became increasingly primer dependent. Hydroxylapatite-chromatographed enzyme preparations were completely dependent upon added dextran for rapid synthesis of methanol-insoluble glucan from sucrose. Half-maximal stimulation of new dextran synthesis occurred with dextran at a concentration of 2 to 3 μM and with a molecular weight of about 2,600. Neither glycogen, amylose, inulin, nor isomaltose functioned as primer. Studies with the dextransucrase activities detectable by in situ assay in polyacrylamide gels subjected to electrophoresis under nondenaturing conditions revealed that the major activity was detectable in the presence of sucrose alone and was stimulated by addition of primer dextran. The minor activity was only detected when primer dextran was present. Homogeneous preparations of both enzymes contained 30 to 40% carbohydrate.

In a previous report (6) we described a purification procedure for the dextransucrase (EC 2.4.1.5) activity from *Streptococcus mutans* strain 6715. Studies with purified enzyme demonstrated that the Michaelis contant for sucrose was 3 mM and that fructose competitively inhibited the enzyme reaction ($K_i \sim 27$ mM). Polyacrylamide gel electrophoresis and in situ assay of highly purified enzyme preparations revealed the presence of two activities in the approximate ratio of 1:10. During these studies we noted (6) that in order to obtain rapid dextran synthesis with purified enzyme preparations, a primer dextran must be included in the reaction mixture. Although other investigators have briefly mentioned the stimulating effect of added dextran on the reaction rate of the dextransucrase from *S. mutans* (16) and *S. sanguis* (5, 19), this observation has not been further analyzed. In this communication we describe the effects of primer dextran on enzyme preparations at various stages of purification and report on the stability, primer size requirement, and carbohydrate content of this enzyme.

MATERIALS AND METHODS

Bacteria and media. *S. mutans* strains 6715 and GS-5 were obtained from the Forsyth Dental Center, Boston. Strain S-19 is a mutant of 6715 that produces elevated levels of dextransucrase. This variant was isolated in our laboratory from an ultraviolet-treated culture. Bacteria were grown anaerobically in Trypticase soy broth (BBL, Cockeysville, Md.) supplemented with 0.5% yeast extract (Difco, Detroit, Mich.), 2% glucose, and 0.1 M KPO_4 buffer, pH 6.8 (6). Cell-free culture supernatant fluids were prepared as before (6).

Dextransucrase assay and purification. Enzyme activity was estimated by measurement of the synthesis of radioactive methanol-insoluble dextran from [U-^{14}C]sucrose as previously described (6). Reaction mixtures contained (final concentrations): 50 mM sodium acetate buffer, pH 5.5; 7 mM NaF; 20 mM total sucrose containing about 7 mM [U-^{14}C]sucrose (3.35 Ci/mol); and a source of enzyme. Primer dextran was included in the appropriate situations at the concentrations given in the text. Under our conditions, levansucrase activity in culture supernatant fluids of strains 6715, S-19, and GS-5 is only detected after 4 h of incubation (unpublished data), thus, since our experiments with culture supernatant fluids were of short duration (up to 2 h), radioactive polymers that were synthesized were exclusively dextran. Purification of dextransucrase from culture supernates of glucose-grown cells involved ammonium sulfate precipitation, hydroxylapatite chromatography, and isoelectric focusing (6). The ammonium sulfate step separated dextransucrase from levansucrase (6). Polyacrylamide gel electrophoresis and assay of dextransucrase in such gels has already been described (6).

Protein was measured according to Lowry et al. (15) by using bovine serum albumin as a standard. The carbohydrate content of dextransucrase preparations was estimated by the method of Dubois et al. (7).

Isomaltodextrins. Full details of the partial acid hydrolysis and paper chromatography of commercial dextran will be described elsewhere (G. Germaine, C. Schachtele, and A. Chludzinski, J. Dent. Res., in press). Briefly, dextran at 50 mg/ml was hydrolyzed for 45 min at 100 C in 0.3 N H_2SO_4 (28) and the products were separated by column chromatography (2.6 by 70 cm) on G-25 Sephadex (fine) using water as eluant. Samples (15 μliters containing 8 to 13 μg of reducing equivalents) of the Sephadex fractions were applied to Whatman no. 1 paper and developed with a descending flow of ethylacetate:pyridine:water (10:4:3, vol/vol) (29). Sugars were visualized with a $AgNO_3$-NaOH spray reagent (12). The degree of polymerization (DP, glucose residues/isomaltodextrin molecule) of isomaltodextrins was determined by comparison of total hexose residues (7) and reducing residues (22).

Chemicals. Dextran (Fraction T10) and Sephadex were purchased from Pharmacia, Upsalla, Sweden. Glycogen and amylose were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wisc. Sigma Chemical Co. (St. Louis, Mo.) and General Biochemicals (Chagrin Falls, Ohio) were the source of isomaltose and inulin, respectively. [U-^{14}C]sucrose (3.35 Ci/mol) was supplied by New England Nuclear Corp., Boston, Mass.

RESULTS

Dextran stimulation of dextransucrase activity in culture broths. Dextran synthesis by dextransucrase in culture supernatant fluids of two strains of *S. mutans* is shown in Fig. 1. In the absence of added dextran (solid circles) the S-19 and GS-5 dextransucrases polymerized 25 and 11 pmol of sucrose-derived glucose per min, respectively. Inclusion of dextran (167 μg/ml) in the reaction mixtures increased the rates of synthesis (open circles) to 82 and 22 pmol per min in strains S-19 and GS-5. Dextran stimulation of enzyme activity has also been observed in *S. mutans* strains Ingbritt, 10449, LM7, and B2 (unpublished data). Hereafter, enzyme active in the absence of primer dextran will be referred to as dextran-independent activity. Accordingly, primer-stimulated activity is denoted as dextran-dependent.

Storage stability of unpurified dextransucrase. Culture fluids containing dextransucrase activity were stored at 4 C in order to evaluate the stability properties of unpurified enzyme. When the enzyme activity was assayed in the absence of added dextran, it appeared the enzyme from both strains S-19 and GS-5 suffered a substantial loss in activity over 97 days (Fig. 1). The rates of glucose polymerization had decreased from 25 and 11 pmol per min (solid circles) to 10 and 2 pmol per min (solid triangles) for S-19 and GS-5 enzyme, respectively. In marked contrast, when the culture fluids were assayed in the presence of primer dextran, the results indicated dextransucrase was completely stable to storage (open triangles). These data suggest dextran primer was present in the original culture fluids and accounted for the dextran-independent activity.

Dextran stimulation of purified dextransucrase. Ammonium sulfate precipitation of dextransucrase from strain 6715 culture broths, followed by dialysis, preserved dextran-independent enzyme activity (open circles, Fig. 2a). Dextran-independent activity, however, was lost upon passage through hydroxylapatite (HA) (Fig. 2b). HA enzyme was almost totally dependent upon the presence of dextran for activity (solid circles, Fig. 2b). Dextran-independent activity was not restored by including in the reaction mixtures the other protein fractions (singly or in combinations) which were eluted from the same column. HA enzyme was then subjected to isoelectric focusing (IEF) and dialyzed (6). Final purification was over 1,500-fold. Enzyme activity is shown over an extended period of time (6 h) in Fig. 2c. Note that dextran synthesis in the absence of primer (open circles) was eventually detected but proceeded at a very low rate. The inset to Fig. 2c is a replot of the enzyme activity assayed in the absence of exogenous dextran on an expanded ordinate scale. Note the apparent autocatalytic increase in the rate of synthesis, especially after 3 h. This may reflect auto-priming of activity by endogenously synthesized dextran (i.e., product). In some enzyme preparations, the apparent auto-priming commenced after 1 to 2 h of incubation and increased exponentially (unpublished data). The rate of dextran synthesis, however, never attained the exogenously primed rate.

Stimulation of dextransucrase activity is limited to dextran (Table 1). Other glucans (glycogen and amylose) and a fructan (inulin) were without effect when tested with HA enzyme. Isomaltose was similarly ineffective. Maximal stimulation of activity required from 2×10^{-5} to 6×10^{-5} M dextran (molecular weight of 10,000) with 50% of maximal stimulation occurring at 2×10^{-6} to 3×10^{-6} M dextran (Fig. 3).

S. mutans dextransucrase preparations contain two activities which were detected by polyacrylamide gel electrophoresis followed by in situ assay (6). The major activity accounted for 75 and over 90% of the total activity in HA and IEF preparations, respectively. In situ

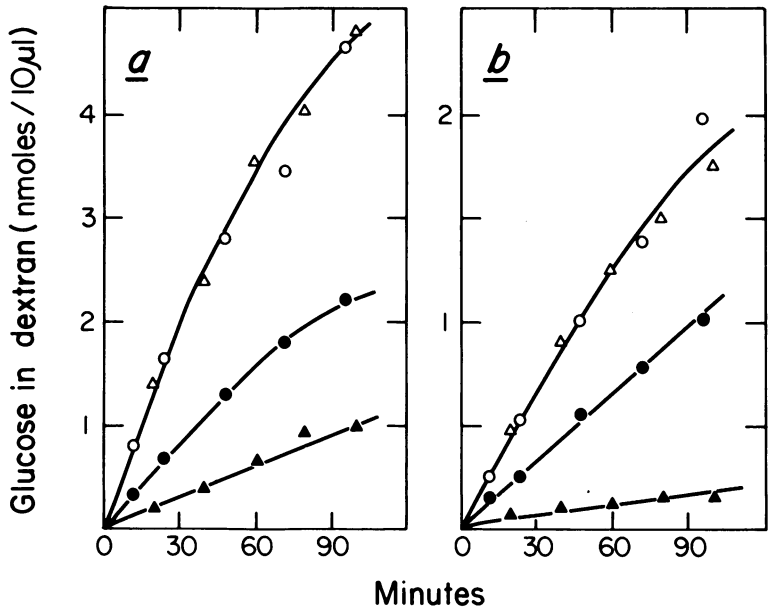

FIG. 1. *Effect of exogenous dextran on activity of dextransucrase in fresh and stored culture fluids. Strains S-19 (part a) and GS-5 (part b) were grown to an optical density (660 nm) of 0.6 to 0.7 and the cells removed by centrifugation. Culture fluids were supplemented with 200 µg of NaF per ml and stored at 4 C. Dextransucrase activity was determined after storage for zero (○, ●) and 97 (△, ▲) days in the present (17 µM) (○, △) or absence (●, ▲) of dextran.*

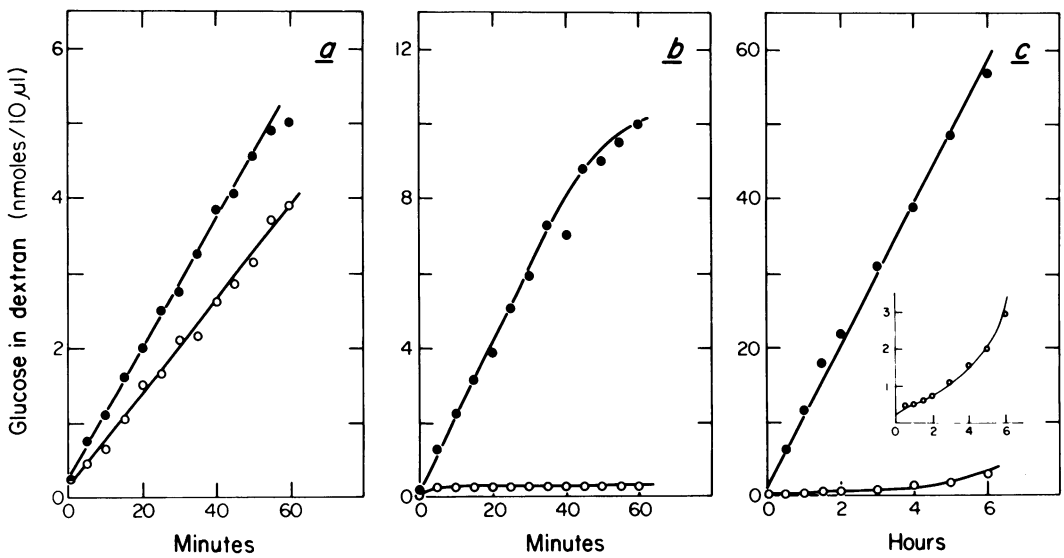

FIG. 2. *Effect of exogenous dextran on dextransucrase activity during enzyme purification. Dextransucrase from strain 6715 was assayed in the presence (●) and absence (○) of dextran (17 µM) after each step of enzyme purification (6): (a) ammonium sulfate precipitation; (b) hydroxylapatite chromatography; (c) isoelectric focusing. Final protein concentration assayed: (a) 1 mg per ml; (b) 0.12 mg per ml; (c) 0.09 mg per ml.*

assay in the absence of primer dextran fails to detect the minor enzyme activity in either HA or IEF preparations (curves 3 and 4, Fig. 4). Assay in the presence of exogenous dextran clearly shows both enzymes (curves 1 and 2, Fig. 4). Thus both the major and minor dextransucrase activities are stimulated by dextran.

Dextran primer size requirement. Commercial dextran (molecular weight 10^4) was partially acid-hydrolyzed and chromatographed on

TABLE 1. *Specificity of stimulation of dextransucrase*

Compound tested	Concn[a]	Glucose[b] in dextran (nmol per 60 min per ml)
None		7.5
Dextran	200 μg/ml	224.6
Glycogen	20 mg/ml	10.8
Amylose	3 mg/ml	9.3
Inulin	200 μg/ml	7.9
Isomaltose	6.8 μg/ml	9.0

[a] Added to give about 2×10^{-5} M.
[b] Sucrose-derived glucose.

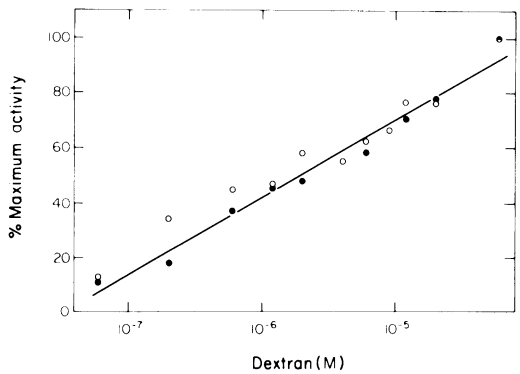

FIG. 3. *Effect of dextran concentration on dextransucrase activity. Isoelectric focused enzyme was assayed at final protein concentration of 66 μg per ml (●) and 80 μg per ml (○) in the presence of saturating (20 mM) sucrose and varying dextran concentrations for 20 min at 37 C.*

G-25 Sephadex to effect a partial separation of the isomaltodextrin products. The predominant isomaltodextrin present in each fraction was estimated by paper chromatography. Chemical chain length determinations on the Sephadex fractions indicated that heterogeneity commenced with fraction 170 (Table 2). This fraction contained predominantly isomaltooctaose (as judged by paper chromatography) but also had sufficient amounts of higher isomaltodextrins to give an average DP of about 12. The priming ability of each fraction was estimated with GS-5 enzyme (Fig. 5). Primer activity commenced with a DP of about 8. The level of priming activity shown by unhydrolyzed dextran was attained by the fraction with a DP of 23. The final concentrations of added carbohydrate from each Sephadex fraction in the reaction mixtures were all in 10- to 50-fold excess (Table 2) as judged by the saturation levels for unhydrolyzed dextran (Fig. 3). The failure to observe stimulation of dextran synthesis with isomaltohexaose and isomaltopentaose is not due to the assay procedure since the dextransucrase assay we use will detect isomaltopentaose (G. Germaine, C. Schachtele, and A. Chludzinski, J. Dent. Res., in press).

Carbohydrate content of dextransucrase. The rather extreme stability of dextransucrase to extended storage (Fig. 1) and recent reports that modestly purified enzyme preparations contained substantial (40%) carbohydrate (J. E. Ciardi and C. L. Wittenberger, p. 188, Bacteriol. Proc., 1973; W. R. Scale, J. Mazza, and J. R. Edwards, p. 188, Bacteriol. Proc., 1973) prompted us to look for carbohydrate in our most highly purified preparations. HA enzyme was purified to homogeneity by a small scale preparative polyacrylamide gel procedure (Table 3). The major and minor activities were completely separated from each other and from other proteins (as judged by re-electrophoresis in polyacrylamide followed by in situ assay). The preparative polyacrylamide gel enzymes contained 32 to 38% carbohydrate (Table 3). Preparations subjected to either HA chromatography alone or both HA and IEF also contained large amounts of carbohydrate suggesting that many of the extracellular proteins elaborated by *S. mutans* may be glycoproteins.

DISCUSSION

The data in Fig. 1 and 2 suggest that sufficient quantities of dextran are present in culture supernatant fluids from glucose-grown *S. mutans* to prime 30 to 70% of the dextransucrase activity that is present. Although sucrose was not included in the culture medium we suspect that sufficient quantities were present to allow limited dextran synthesis to occur during growth. This notion is supported by our repeated observation of mild aggregation of strain 6715, and its derivative S-19, upon inoculation into fresh culture media. (Dextran causes aggregation of *S. mutans*, see ref. 20). Aggregation occurred over the first hour or so and eventually was not discernible as the culture mass increased. Two recent reports (17, 20) have also indicated that glucose-grown *S. mutans* cultures contain small amounts of dextran. It was suggested that both tryptic soy broths (20) and glucose (17) contained low levels of sucrose. We have also found that yeast extract may contain sucrose, since aggregation of freshly inoculated cultures was reduced when this component was omitted from our medium (unpublished data).

S. mutans dextransucrase is stable to extended storage as evidenced by assay in the presence of primer dextran (Fig. 1). Thus, the apparent decrease in enzyme activity, as evi-

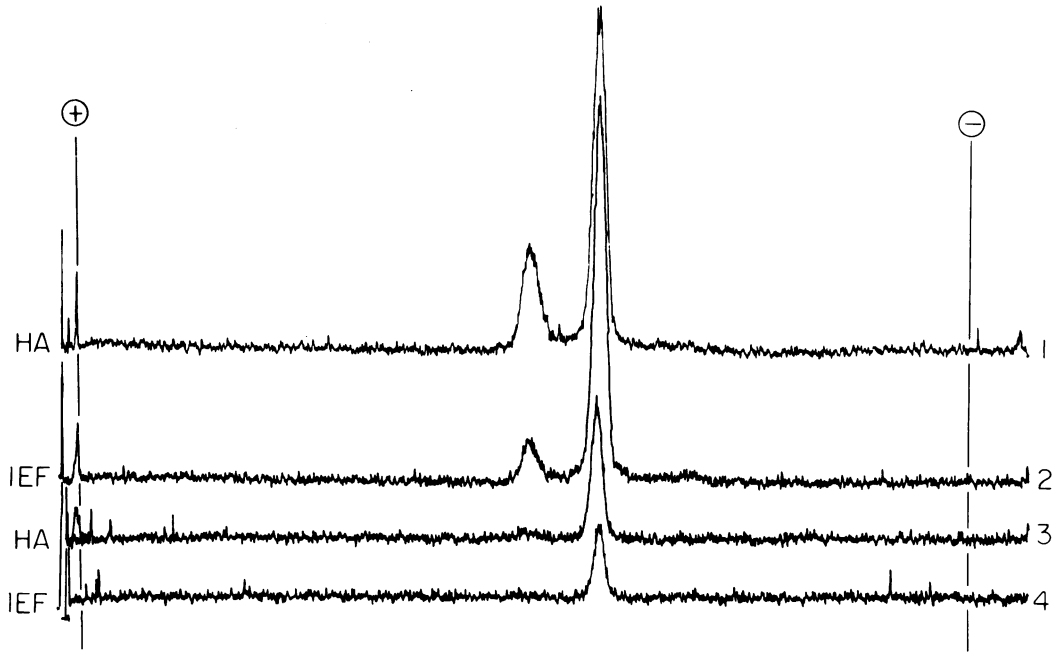

FIG. 4. *Autoradiographic assay of dextransucrase in polyacrylamide gels. Gels containing HA (numbers 1 and 3) and IEF (numbers 2 and 4) enzyme samples were incubated with [U-^{14}C]sucrose as before (6) in the absence (numbers 3 and 4) and presence (numbers 1 and 2) of 17 μM dextran for 3 h at 37 C. Migration of proteins was from right to left. Profiles are microdensitometer tracings of developed autoradiographs of product dextran.*

TABLE 2. *Estimation of size of isomaltodextrin fractions from G-25 Sephadex chromatography*

Fraction	Chain length[a]		Concn in assay (mM)[b]
	Chromatographic	Chemical	
130	—[c]	30.8	0.83
140	—	23.0	1.04
150	—	17.7	1.07
160	—	15.7	1.05
170	8	12.2	0.90
180	7	8.4	1.00
190	6	7.1	0.94
200	5	6.4	0.75
210	4	3.3	0.90
220	3,2	2.8	0.90
230	2	2.4	1.22
260	1	1.7	0.85

[a] Glucose residues per isomaltodextrin molecule.
[b] Refers to assays of Fig. 5.
[c] A dash indicates not possible to estimate.

denced by assay in the absence of primer dextran, clearly is not a result of loss of dextransucrase but rather most probably due to the storage instability of endogenously present dextran which acted as primer. Since many *S. mutans* strains, including 6715 and GS-5, produce a dextranase activity during growth on glucose (23), storage instability of such endogenous priming dextrans may result from degradation by dextranase to a size which precludes functioning as a primer.

When the *S. mutans* dextransucrase is precipitated with ammonium sulfate and passed through HA the enzyme becomes primer dependent (Fig. 2b). As mentioned previously (6), HA is known for its ability to adsorb dextrans (21) and thus this step in purification probably removes dextran which was previously associated with the enzyme.

Analysis of the homogeneous dextransucrase preparations from polyacrylamide gels (Table 3) revealed the presence of 32 and 38% carbohydrate in the major and minor enzyme bands. Since it is clear that the HA step removed dextran capable of functioning as primer (Fig. 2), our results are in agreement with the recent suggestion of Scales, Mazza, and Edwards (Bacteriol. Proc. 1973, p. 188) that carbohydrate detected in *S. mutans* dextransucrase is not dextran. We have not as yet obtained sufficient quantities of the major and minor enzyme activities to identify the carbohydrate portion of the enzyme and to determine whether the carbohydrate is actually covalently attached to the enzyme or simply just carried through the purification procedure.

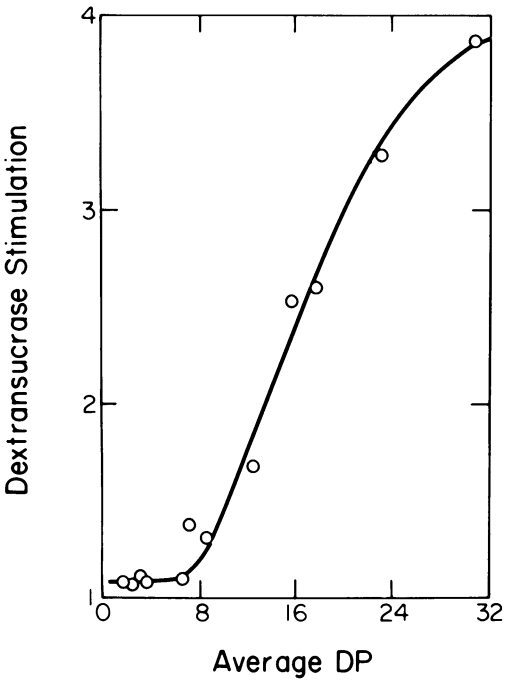

FIG. 5. *Effect of dextran molecular weight on primer activity. The Sephadex fractions listed in Table 2 were added to assay mixtures containing primer dependent S. mutans strain GS-5 dextransucrase and incubated for 60 min. Ordinate: ratio of new dextran synthesis with/without primer dextran. Abscissae: chemically determined degree of polymerization (DP). Unhydrolyzed dextran (25 μM) gave a 3.42-fold stimulation of activity.*

Dextran stimulation of either unpurified or modestly purified dextransucrase from bacteria other than *S. mutans* (16) and *S. sanguis* (5, 19) has been known for some time (8, 9, 18, 24, 25). The enzyme from *Leuconostoc mesenteroides* is most completely characterized (9, 18). Exogenous dextran is thought to act as a primer (acceptor) and thus to become covalently bonded to newly synthesized dextran (4, 8). In the presence of primer dextran, initial synthesis is rapid. When only sucrose is present, initial synthesis is very slow (4); sucrose must act as both glucosyl donor and acceptor. The apparent affinity of the *S. mutans* dextransucrase for dextran is about 1,000-fold higher than it is for sucrose. This follows from a comparison of the K_m values for sucrose (3 mM [6]) and dextran (estimated to be about 3 μM from Fig. 3 and unpublished data). It is apparent with the *S. mutans* enzyme that sucrose may act as a glucosyl acceptor in the absence of added dextran (Fig. 2c). As evidenced from the long lag, however, sucrose is an inefficient acceptor and devoid of priming activity. Given enough time, the products of the reaction attain sufficient sizes to act as primers themselves (auto-priming). It has recently been shown that dextransucrase from *S. mutans* OMZ176 can utilize isomaltodextrins (DP 2-7) as glycosyl acceptors (26, 27). In the experiment presented in Fig. 5, we attempted to assess the relative priming ability of isomaltodextrins. In order to minimize opportunities for auto-priming by products derived from initially nonpriming glucosyl acceptors, we used a short incubation period (60 min). Stimulation of dextransucrase commenced with isomaltooctaose and reached the level exhibited by unhydrolyzed dextran (DP *ca.* 60) in the fractions with a DP of 23.

Dextrans produced by *S. mutans* are made up of α-(1 → 6)-linked glucose molecules with α-(1 → 3)-linked branch points (10, 11, 13, 14). Highly-branched dextran exhibits poor aqueous solubility and is usually present only after extended reaction periods (11; unpublished data). Previous workers have suggested that a single enzyme catalyzes the synthesis of both α-(1 → 6) and α-(1 → 3) linkages (8, 9) since only one enzyme was discernible throughout purification. Other reports have described up to seven activities from *S. mutans* (10). Multiple dextransucrase peaks following HA and IEF procedures have not been seen by us. We have

TABLE 3. *Carbohydrate content of dextransucrase fractions during purification*

Fraction	Protein (mg/ml)	Carbohydrate (mg/ml)	Carbohydrate (%)
Hydroxylapatite chromatographed	3.0	1.6	35[a]
Isoelectric focused	1.0	0.69	41
Slab polyacrylamide gel electrophoresed[b]			
A. Major band[c]	0.054	0.025	32
B. Minor band[c]	0.044	0.027	38

[a] Percent carbohydrate calculated on the basis of total weight (protein + carbohydrate) for each fraction.

[b] Hydroxylapatite fraction used for electrophoresis.

[c] Enzyme bands were localized by in situ radioassay of a test strip cut from the extreme edge of the gel. Proteins were visualized with Coomassie blue on a similar strip. Areas of the remainder of the slab gel which contained the major and minor enzyme activities were dissected from the rest of the gel and from each other. Strips containing each enzyme activity were macerated by passage through an 18-gauge needle. Enzyme was eluted from each macerated gel strip with 0.05 M Na acetate buffer, pH 5.5, for 48 h at 4 C.

consistently observed only two enzyme activity bands in polyacrylamide gels of either HA or IEF enzyme preparations (6), both of which are stimulated by dextran (Fig. 4). Although linkage analyses of the product(s) of either enzyme has not been done (due to the lack of sufficient quantities of homogeneous major and minor enzyme), we suspect the two separated activities represent a α-(1 $\rightarrow$ 6) transferase and a α-(1 $\rightarrow$ 3) branching enzyme. (In this context, branching activity is not meant to imply transfer of an oligosaccharide to an acceptor polysaccharide to create a branched product, but rather the transfer of a single sucrose-derived glucosyl residue to a predominantly α-(1 $\rightarrow$ 6) acceptor dextran to form a α-(1 $\rightarrow$ 3) branch point one residue long.) If branching (α1 $\rightarrow$ 3) and transferase (α1 $\rightarrow$ 6) activities reside in distinct enzymes, one might expect the branching enzyme to exhibit an absolute requirement for dextran as a substrate (acceptor). Transferase, on the other hand, could build linear dextrans by either adding on to primer (acceptor) dextran or acceptor sucrose. Since sufficient enzyme protein is present in the minor band of HA preparations (see Fig. 3 of ref. 6) to readily allow detection of dextran-independent transferase activity by in situ assay, and since enzyme activity is only observed in the minor band in the presence of primer dextran (Fig. 4), it appears possible that the minor band contains an enzyme that can only synthesize (α1 $\rightarrow$ 3) branch points.

ACKNOWLEDGMENTS

We wish to thank Betty Thorpe for technical assistance.

This work was supported by Public Health Service grant DE-03654 from the National Institute of Dental Research. C.F.S. was the recipient of Public Health Service Career Development Award K4-DE-42,859 from the National Institute of Dental Research.

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