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The Effect of Dextranase on Dextran Production by Streptococcus Mutans 6715 S-19 Glucosyltransferase

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THE EFFECT OF DEXTRANASE ON DEXTRAN PRODUCTION BY *STREPTOCOCCUS MUTANS* 6715 S-19 GLUCOSYLTRANSFERASE

by

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DEDICATION

To my father, Joseph E. Nonnenmann,
who not only taught me how to work
hard, but also how to enjoy it.

ACKNOWLEDGMENTS

I would like to express my deep appreciation to the following people

To Andrew Chludzinski, Ph.D., who started as my research advisor and became one of my closest friends. The knowledge he gave me is a gift that will never be equaled by any other friend.

To John V. Madonia, D.D.S., Ph.D., a man who believed in me and gave me a chance.

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VITA

The author, Jamie D. Nonnenmann, is the fifth of nine children born to Joseph E. Nonnenmann and Adreine (Martin) Nonnenmann. He was born January 1, 1953, in Rock Island, Illinois.

His elementary education was obtained at St. Joseph Catholic School and secondary education was completed at Alleman High School. He graduated in 1970.

In August, 1970 the author registered at St. Ambrose College, Davenport, Iowa and in 1974 received a Bachelor of Science in Biology. While attending St. Ambrose College he was an officer in Beta Beta Beta Biological Honor Society.

In 1976 he was accepted to Loyola Dental School where he is completing requirements towards a D.D.S. degree.

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CHAPTER I

INTRODUCTION

The dental carious lesion is a decalcification of the hard tooth structure by the acidic metabolic end products of oral microorganisms. The primary etiological agent of this disease is the bacterium Streptococcus mutans which has the ability to produce an extracellular polysaccharide capsule, dextran, which mediates the adherence of this organism to the tooth surface. This capsule dextran functions as the matrix for plaque formation by the aggregation of other organisms and food debris. Streptococcus mutans produces dextran by a constitutive extracellular enzyme system collectively referred to as dextransucrase or glucosyltransferase (E.C. 2.4.1.5). This enzyme system produces both water-soluble α (1,6) linked and water-insoluble α (1,3) linked dextrans by utilizing the glucosyl moiety of sucrose and attaching it to the non-reducing terminus of an acceptor sucrose or small dextran (primer) molecule. This organism also produces another constitutive enzyme, dextranase, which has the ability to hydrolyze α (1,6) linkages in a dextran molecule larger than 10 glucose units. The purpose of the present study is to elaborate on the characteristics of the glucosyltransferase enzyme system and determine the role

of dextranase in the synthesis of both water-soluble and water-insoluble dextran molecules.

CHAPTER II

REVIEW OF THE LITERATURE

The primary cause of tooth decay is the accumulation and subsequent metabolic activity of an amorphous mass of oral bacteria called "plaque", on the surfaces of teeth. Clark[13] first demonstrated that the etiological agent of dental caries was the morphologically variable bacterium, Streptococcus mutans. This species has been shown to be both serologically[5] and genetically[14] heterogeneous, consisting of five biotypes designated a-e. Other major streptococcal species consistently isolated from the oral cavity include; S. sanguis found primarily in plaque, S. salivarius located on the tongue and S. mitis an inhabitant of the oral mucosa.[7] Guggenheim[43] isolated strains of S. mutans and S. sanguis from potentially cariogenic plaque. Using gnotobiotic hamsters and rats, he demonstrated that S. mutans induced a carious lesion but S. sanguis, although forming plaque, showed much less caries activity. These S. mutans strains were classified as HS and FA strains and were identical morphologically to the organism described by Clark. DeStoppelaar[17,60] also demonstrated that there was a direct relationship between the occurrence of "caries inducing streptococci" from a human carious lesion and

an increase in caries incidence in hamsters. Fitzgerald[30] found that gnotobiotic animals monoinfected with S. mutans produced plaque with a carious lesion while S. sanguis and S. salivarius formed plaque but did not produce a carious lesion. Other studies have also corroborated that S. mutans is the "caries inducing organism".[26,18,27]

It was observed clinically that plaque accumulation occurs more readily under specific dietery environmental conditions.[89] An increase in plaque formation was demonstrated when animals were placed on a high sucrose diet.[6] This plaque was found to contain a sticky dextran-like polymer.[38] Gibbons[39] using S. mutans monoinfected rats, showed that this organism was primarily localized in the occlusal fissures of molar teeth, when placed on a high sucrose diet. Mutants of cariogenic S. mutans which do not produce this sticky or branched polysaccharide in plaque[19] had lost the ability to adhere to glass culture flasks[56] and were shown to have a low caries score in hamsters and rats. However, cellular agglutination with cariogenic S. mutans 6715 was observed in the presence of either linear or branched high molecular weight dextran.[40]

Plaque contains a variety of polysaccharides, which can be designated into three types, intracellular, cell structural, and extracellular.[96] Formation of plaque depends on the production and presence of the extracellular polysaccharide, dextran.

Levans produced by cariogenic streptococci, however, are involved as metabolites, while dextrans demonstrated a matrix function.[73] Polysaccharide of a dextran nature was shown to be produced enzymatically from sucrose by Leuconostoc,[50] and Streptococcus bovis,[28] while S. salivarius produced levans.[95] Cariogenic streptococci produced a glucose containing polysaccharide[83] which contained 80% glucose and 18% fructose.[16] Gold[42] found that after sucrose rinses for three days, human plaque increased in total hexose to 1% dextran and 3% levan. Sidebotham[82] postulated three roles for these polysaccharides in the etiology of dental caries: i) a stable matrix, ii) a diffusion restrictive barrier enabling low pH to be maintained, iii) a source of fermentable carbohydrate. This environment selects for other aciduric organisms such as species of Lactobacilli, an organism long related to the dental caries experience.[41]

Hotz[55] found two types of glucose polysaccharides in pooled plaque, water soluble and water insoluble. Plaque dextran, produced by cariogenic S. mutans was found to contain α (1,6) linkages in the soluble fraction and both α (1,6) and α (1,3) in the insoluble fraction.[63] Levans contained primarily β (1,2) linkages.[3] Freedman[29] studied mutants of S. mutans 6715 and found that those which lost the ability to produce insoluble polysaccharide were unable to form adhesive deposits on wires. However, these organisms did exhibit the ability to produce water soluble glucans and cellular agglutination was not affected. Nalbundian[70] studied

the structure of the cellular capsule of cariogenic S. mutans 6715 with the electron microscope. He demonstrated that adherence did not occur in the absence of a fibrillar glucan normally loosely associated with the cell, but aggregation was not affected when a close, cell associated fibrillar glucan was present.

Streptococcus mutans synthesizes a constitutive, extracellular, dextransucrase which has the ability to hydrolyze sucrose and form the glucose polymer dextran. This enzyme is a member of the class of transglycosylases, designated as (1,6)-α-D glucose: D fructose-2-D glucosyltransferase (E.C. 2.4.1.5.).[82] Initial work with this enzyme dealt primarily with the preparation of dextrans for use as a blood plasma substitute.[58] Dextransucrase was first shown to exist in the supernatant fluids from species of Leuconostoc.[90,88,51] Kobayaski[57] showed a series of isoenzymes both extracellular and intracellular from L. mensteroides after separation on DEAE cellulose. Two enzyme activities were isolated from Betacoccus arabinosaccous by ethanol precipitation,[1] while only one activity could be found in an ammonium sulfate preparation from S. bovis.[2] Carlson[8] using hydroxylapatite chromatography and isoelectric focusing, found only one peak of enzyme activity from S. sanguis. This preparation was responsible for an insoluble glucan with fifty percent α (1,6) linkages. The dextran produced by dextransucrase has been noted to differ in its structure depending on the source of the enzyme.[52] It was

also demonstrated that dextransucrase produced by S. mutans existed in multiple forms.[79] Guggenheim[46] found seven glucosyltransferase activities from an HS strain of S. mutans. After paper electrophoresis, Cybulska[15] demonstrated five to seven protein fractions with only three peaks having dextransucrase activity. It was found later by Chludzinski[10] that only two distinctly active enzymes existed after gel electrophoresis. These results were verified by Fukii[33] and Mukasa[68] who showed that one enzyme fraction formed insoluble glucan and produced adherence while the other enzyme fraction formed mainly water soluble glucan and no adherence. Characterization of two dextran forming activities from S. mutans GS-5 showed separate water insoluble and soluble dextran forming dextransucrases after hydroxylapatite chromatography and gel filtration.[62] S. mutans FA-1 also was shown to produce two dextransucrase activities by cellulose and gel filtration.[76] A cell associated dextransucrase activity from S. mutans GS-5 was demonstrated to exist in both forms,[61] an intracellular and a cell-bound form which was distinct from the extracellular enzyme.

Plaque formation has been shown to require the synthesis of water insoluble polymer by cell-bound enzymes and the participation of a binding site on the cell surface.[66] This binding site was not protein,[72] but of a dextran nature that binds enzyme allowing cellular adherence to glass.[67] McCabe[65] postulated three steps in dextran formation: i) enzyme is reversibly

bound to dextran, ii) enzyme is irreversibly bound by physical entrapment, iii) enzyme is making insoluble dextran until inactivated. Germaine[34] demonstrated that the presence of endogenous dextran increased the activity of dextransucrase allowing rapid product-dextran formation to occur, thus showing dextransucrase to be "primer-dependent". The mechanism of synthesis of dextran was once postulated to be the addition of glucose units to the reducing end of the acceptor chain.[75] Now however, synthesis is thought to be the addition of glucose units to the non-reducing terminus of primer, acceptor dextran and more than one enzyme molecule can bind simultaneously to the high molecular weight dextran.[36,24,11]

Since the plaque matrix consists primarily of dextran, dextranase enzymes were examined as a possible therapeutic agent in plaque removal and prevention. Dextranase has been widely found in nature having been isolated from plants,[54] soil organisms,[74] gram negative intestinal bacteria,[53] molds,[91] and in the genus Bacteroides.[81] These enzymes consist of α (1,6) glucan-6-glucanohydrolases and α (1,3) glucan-3-glucanohydrolases which hydrolyze α (1,6) and α (1,3) linkages respectively in the dextran molecule. Dextranases can be further classified into two types, according to the portion of the dextran molecule that they hydrolyze. The endo-type acts on the interchain linkages while the exo-type acts on the end residues of the molecule.[22]

It was originally shown that hamsters given mold dextranase in drinking water showed a decrease in plaque formation.[49,31] Rats however, showed either a decrease[45] or no effect[59] in plaque accumulation when given dextranase while on a high sucrose diet. Fitzgerald[32] also showed a decrease in hamster and rat plaque but human plaque was not affected when tested with three different dextranase preparations from broth cultures of Penicillium funuculosum. Penicillium dextranase was again shown to be effective in reducing plaque in S. mutans monoinfected rats. However, when normal oral flora was present, no decrease was observed in plaque formation.[47] Guggenheim[48] demonstrated that insoluble dextran polymerized in vitro by dextransucrase was inhibited by a fungal dextranase. Spicaria violaceae dextranase was shown to be effective in removing dental plaque in humans when administered as a rinse.[69] The variability in the hydrolysis of plaque dextran is due to a variable proportion of α (1,3) linkages to α (1,6) linkages. It was concluded that the more α (1,3) linkages present in the native dextran molecule, the less susceptible these dextrans are to hydrolysis by mold dextranases.[4,9,71] However, it was found that a dextranase preparation from Cludosporium resinae contained three enzymes capable of degrading dextrans.[20] One of these enzymes, an α (1,3) glucanase hydrolyzed an insoluble glucan produced by cariogenic S. mutans OMZ-176.[21]

Plaque organisms were investigated in order to determine if they produced extracellular dextranase.[85] Among those studied thirteen defined S. mutans strains were found to produce detectable amounts of enzyme activity.[86] Guggenheim[44] isolated an endo α (1,6) glucan-6-glucanohydrolase activity from cariogenic S. mutans OMZ-176 by hydroxylapatite, BioGel, and DEAE cellulose chromatography. Dextranase preparations from oral strains of Actinomyces and Bacteroides were equally effective in blocking polymer production and adherence of S. mutans in vitro.[80] Dextranase produced by Actinomyces israellei exhibited an endohydrolytic action on α (1,6) linked dextran indicating an antagonistic function towards S. mutans colonization.[87] Walker[93] demonstrated that dextranase isolated from S. mitis released only D-glucose, which indicates an exohydrolytic function. Ebisu[25] showed a predominately α (1,3) linked glucan synthesized by only cariogenic S. mutans in the presence of a Spicaria violacceae dextransucrase preparation. This glucan did not possess the adhesive qualities of the dextran produced in the absence of dextranase. It was concluded that S. mutans' dextranase function was involved in the release of endogenous primer for continued dextran synthesis.

CHAPTER III

MATERIALS AND METHODS

A. Maintenance of Cultures:

Streptococcus mutans strain S-19, an ultraviolet mutant of S. mutans 6715, which produces elevated levels of glucosyltransferase,[77] was obtained from Dr. G. R. Germaine, U. of Minnesota. This organism was grown overnight in Todd-Hewitt broth (BBL, Cockeysville, Md.) at 37°C. Cells were centrifuged at 4000 X g at 4°C for 20 minutes, resuspended in 2% sterile skim milk media (BBL), and lyophilized. Cultures were routinely maintained on Mitis-Salivarius agar plates (Difco, Detroit, Mich.), with biweekly transfers. Fresh cultures were obtained from the lyophilized stock monthly. All cultures were grown under anaerobic conditions (GasPak System, BBL or 80% N, 10% H, 10% CO_2), at 37°C.

B. Growth Media:

Two media were used to grow cells for enzyme purification. The first, TYF media described by Germaine, et al.,[34] consisted of 3% Trypticase Soy Broth (BBL), 2% glucose, 0.5% yeast extract (BBL), 0.1 M potassium phosphate buffer, pH 6.8 and supplemented with 0.5 M fructose. The second media, TYG, contained 3% trypticase (BBL), 0.5% yeast extract (BBL), 2.5% glucose, 1.0 mM

$MnSO_4$, 1.0 mM $MgSO_4$, 0.1 mM $(NH_4)_2SO_4$, 3.2 mM cysteine-HCl, and 0.1 M potassium phosphate buffer, pH 6.8. One liter of cells were grown for 18 hours to an optical density of 0.9 at 540 nm. The cultures were centrifuged at 10,000 X g at 4°C for 30 minutes (Beckman Model J-21, Beckman Instruments, Fullerton, Ca.). The resultant supernatant was used as a source of enzyme for further purification.

C. Ammonium Sulfate Precipitation:

Supernatant proteins from either TYF or TYG media were precipitated at 4°C by adding granular ammonium sulfate with stirring to 55% saturation. This solution was then allowed to stir for an additional 30 minutes and centrifuged at 10,000 X g for 30 minutes at 4°C. The supernatant was discarded and protein pellet was resuspended in 0.01 M potassium phosphate buffer, pH 6.5. This preparation was dialyzed for 24 hours at 4°C against two, two liter changes of the same buffer.

D. DEAE Cellulose Chromatography:

Anion exchange DEAE cellulose (Sigma Chem. Co., St. Louis, Mo.) was washed, equilibrated with 0.01 M potassium phosphate buffer pH 6.5, and degassed according to the methods suggested by the manufacturer. The cellulose was loaded into the column (2.6 X 40.0 cm) and washed with 500 ml of the same buffer. Protein sample (350 mg for TYF and 170 mg for TYG) was added to

the column and washed with 0.01 M potassium phosphate buffer, pH 6.5, until all unbound protein was eluted. A linear gradient of 0.01 to 0.4 M CKl in 0.01 M potassium phosphate buffer, pH 6.5, was used to elute protein at a flow rate of 20 ml per hour and 4 ml fractions were collected. A final wash of 1.0 M KCl in the same buffer eluted any additional protein. All washes and gradients contained 0.001% sodium azide. Protein elution was determined by optical density measurements at 280 nm using a Beckman DB-GT Spectrophotometer (Beckman Instruments, Fullerton, Ca.). Tubes containing dextransucrase activity were pooled and concentrated by dialysis against 20% polyethylene glycol in 0.01 M potassium phosphate buffer, pH 6.5. Purification was monitored by assaying for enzyme activity as described below and by determining total protein by the method of Lowry, et al.,[64] using bovine serum albumin as a standard.

E. Dextransucrase Enzyme Assay:

The assay mixture contained 1.2 mM NaF, 25.0 mM Sucrose, 3.0 μCi per ml [U-^{14}C] Sucrose (3.35 Ci per M, New England Nuclear, Boston, Mass.), 25 μM T_{10} Dextran (Pharmacia Fine Chemicals, Uppsala, Sweden) as a source of primer dextran where indicated, in 0.05 M sodium acetate buffer, pH 5.5. Levansucrase activity was assayed with the same assay mixture except 44.7 μCi per ml of [^{3}H-fructose] sucrose (0.25 Ci per M, New England Nuclear) was present instead of [U-^{14}C] sucrose. Dextransucrase

activity was assayed as described by Germaine, et al.[37] The assay mixture was incubated at 37°C with enzyme and 10λ samples were removed at the times noted. The samples were spotted onto Whatman 3MM filter paper squares, 2 cm X 2 cm, (Reeve Angel, Clifton, N.J.), which were mounted on steel pins and immediately placed into a beaker of absolute methanol (Scientific Products, McGaw Park, Ill.), containing not less than 10 ml per square. All squares were soaked 15 minutes after assay, then batch washed twice in the volume of methanol with a 15 minute soak between washes. The squares were then dried under a heat lamp and placed in glass vials containing 10 ml of scintillation fluid. The scintillation fluid consisted of 100 mg of 1,4-bis [2-(5-Phenyloxazolyl)] benzene (POPOP, Amersham/Searle, Des Plaines, Ill.) and 4 g of 2,5-diphenyloxazole (PPO, J. T. Baker Chem. Co., Phillipsburg, N. J.) per liter of Scintillar (Mallinckrodt, St. Louis, Mo.)[10]. The samples were counted on a Packard Tri-Carb 3320 liquid scintillation spectrometer (Packard Instr. Corp., Downers Grove, Ill.). One unit of dextransucrase activity is that amount of enzyme causing the polymerization of 1.0 µM of sucrose-derived glucose per minute at 37°C[84]. Dextranase-CB (55 Units per mg Calbiochem, LaJolla, Ca.) was added to the reaction mixture at a final concentration of 6 µg per ml where indicated.

F. Preparation of Isomaltodextrins:

Dextran T_{10} (Pharmacia) at a final concentration of 250 mg per ml (20 ml) was boiled in the presence of 0.3 N H_2SO_4 for 60 minutes in a hot water bath in order to hydrolyze the dextran.[23] After boiling the solution was neutralized with 1 N NaOH and a 4.0 ml sample of the isomaltodextrins was put on a Sephadex G-25 (Pharmacia Fine Chem., Piscataway, N. J.) column (2.6 X 47.0 cm), previously equilibrated with distilled water. The isomaltodextrins were eluted from the column with distilled water containing 0.001% sodium azide a room temperature at a flow rate of 18 ml per hr. Fractions of 0.75 ml were collected and every third tube was assayed by the methods of Somogyi[79] and Dubois, et al.,[94] for total hexose and reducing equivalents respectively. The degree of polymerization (DP) of the isomaltodextrins was equal to the number of glucose residues per reducing end in a fraction. Fractions with similar DP were pooled and used as a source of isomaltodextrins. Glucose and Blue Dextran were used as standards to determine the void volume and elution volume of the column.

CHAPTER IV

RESULTS

Streptococcus mutans' S-19 glucosyltransferase was partially purified in order to investigate the enzymatic activity of its multiple species in the absence of culture proteins. TYF and TYG culture supernatants were purified according to the steps outlined in Tables 1 and 2 respectively. Table 1 shows that after ammonium sulfate precipitation and dialysis the TYF preparation increased 38-fold in enzyme purification, with a 35% decrease in total protein and a 21% increase in enzyme activity. Anion exchange column chromatography with DEAE cellulose, followed by dialysis in 25% polyethylene glycol in buffer resulted in a final 97-fold purification with about 25% of the original protein. Enzyme yield was not altered by this procedure. The TYG preparation (Table 2) showed a 24-fold purification of enzyme activity by ammonium sulfate precipitation with a 94% decrease in the protein content of the preparation. Enzyme activity decreased 24% by this procedure. DEAE cellulose chromatography of this preparation resulted in the separation of two peaks of enzyme activity. Enzyme I was purified 133-fold and contained 3% of the original protein, while Enzyme II was purified 237-fold and contained 2% of the original protein. Enzymes I and II combined decreased 13% in enzyme activity by this procedure.

TABLE 1. Purification of glucosyltransferase from TYF culture supernatent of S. mutans 6715 S-19

	Fraction	Volume (ml)	Total Protein (mg)	Total Activity (U)	Specific Activity 10^{-2}U/mg Prot.	Purification (Fold)	Yield (%)
I.	Culture Supernatant	1000	10,800	75	0.69	0	100
II.	Ammonium Sulfate	50	350	91	26	38.0	121
III.	DEAE Chromatographed and concentrated	50	135	90	66.7	97.0	120

TABLE 2. Purification of glucosyltransferase from TYG culture supernatent of S. mutans 6715 S-19

	Fraction	Volume (ml)	Total Protein (mg)	Total Activity (U)	Specific Activity 10^{-2}U/mg Prot.	Purification (Fold)	Yield (%)
I.	Culture supernatant	1000	5600	62	11	0	100
II.	Ammonium Sulfate	50	170	44.5	26.2	24	71
III.	DEAE Chromatographed and concentrated I.	12.5	20	24.3	146.5	133	47
	II.	9.5	9.5	24.8	261	237	40

Table 3. Degree of Polymerization (D.P.) of isomaltodextrins fractions from Sephadex G-25 T_{10} hydrolysate as described in Materials and Methods.

Fraction Number	Elution Volume (ml)	D.P. Ratio	Fraction Number	Elution Volume (ml)	D.P. Ratio
105	140	37.3	155	207	12.2
110	147	27.9	160	213	6.4
115	153	34.5	165	220	6.9
120	160	16.2	170	227	7.9
125	167	14.5	175	233	4.7
130	173	13.8	180	240	4.6
135	180	11.6	185	247	3.5
140	187	11.8	190	253	2.8
145	193	8.7	195	260	2.9
150	200	9.3	200	267	2.5

The DEAE cellulose chromatography elution profile of the TYF ammonium sulfate precipitated culture supernatant proteins (Figure 1) exhibited one peak of glucosyltransferase activity. This peak of activity eluted at 0.06 M KCl and displayed a small shoulder of activity. No other glucosyltransferase activity could be eluted by using a 1.0 M KCl wash. The entire peak of activity (fractions 80 - 90) was pooled and concentrated and used a source of TYF glucosyltransferase for further studies. The TYG ammonium sulfate preparation (Figure 2) was eluted in a similar manner and two peaks of glucosyltransferase activity were observed. The first peak (I) and second peak (II) were eluted from the DEAE cellulose column at approximately 0.06 M and 0.27 M KCl respectively. No other enzyme activity was eluted even with a 1.0 M KCl wash. It was also observed in assaying for the GTF activity of the elution fractions that fractions in peak I produced turbidity in the reaction tubes. This turbidity was absent in assaying TYG II and TYF Enzymes for activity.

Glucosyltransferase is primer dependent, depending on the degree of purification of an enzyme and the presence of endogenous and exogenous dextran. Since the purification of enzyme preparations removed primer, it was necessary to determine the effect of linear dextran T_{10} on the catalytic activity of the different enzyme preparations. Figure 3 demonstrates the effect of dextran T_{10} in priming enzyme from both the TYF and TYG

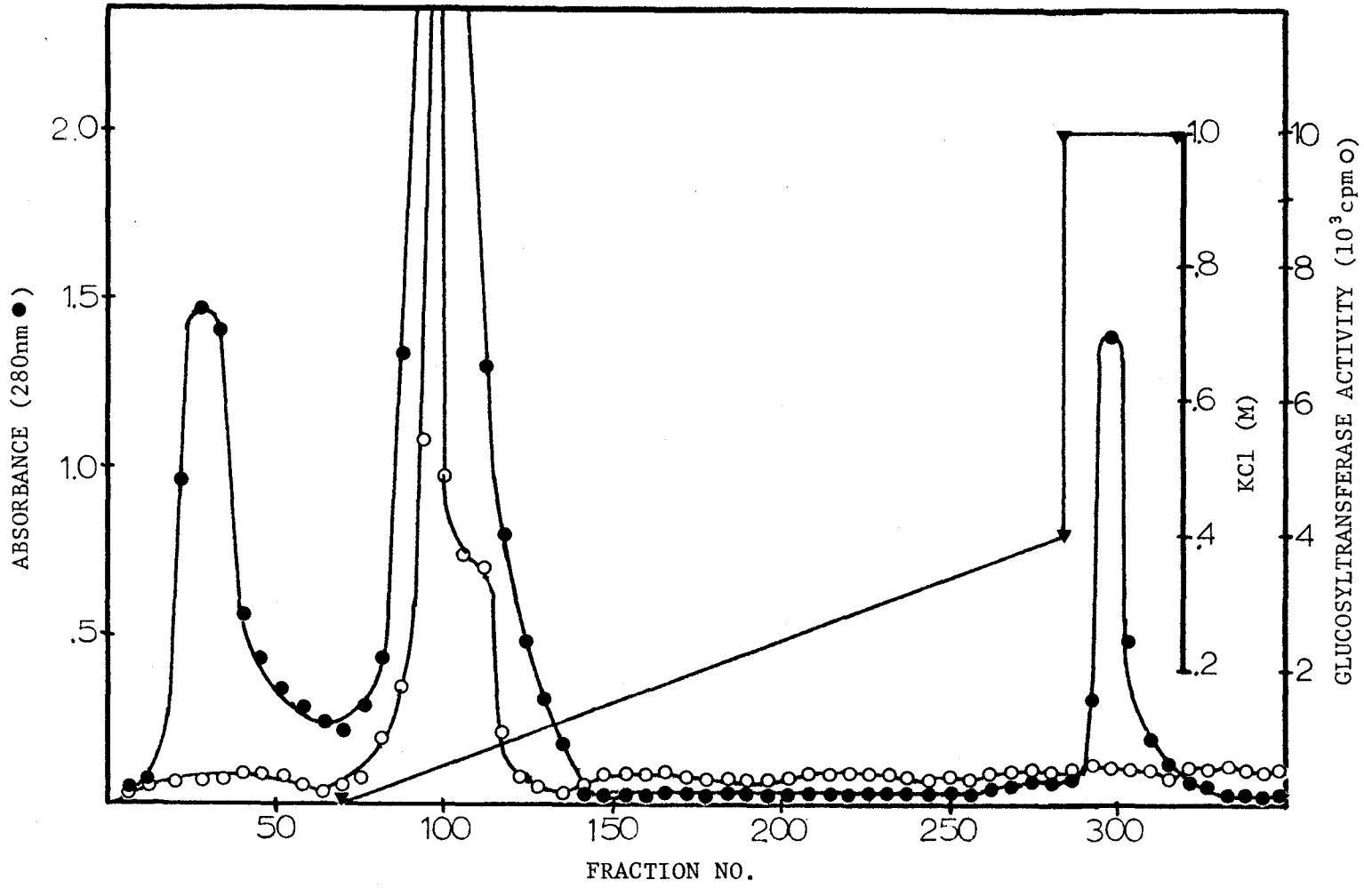

FIGURE 1. DEAE cellulose chromatography of ammonium sulfate precipitated and dialyzed TYF glucosyltransferase. Enzyme activity expressed as the counts (^{14}C) hexose polymerized in 20 min. under standard assay conditions.

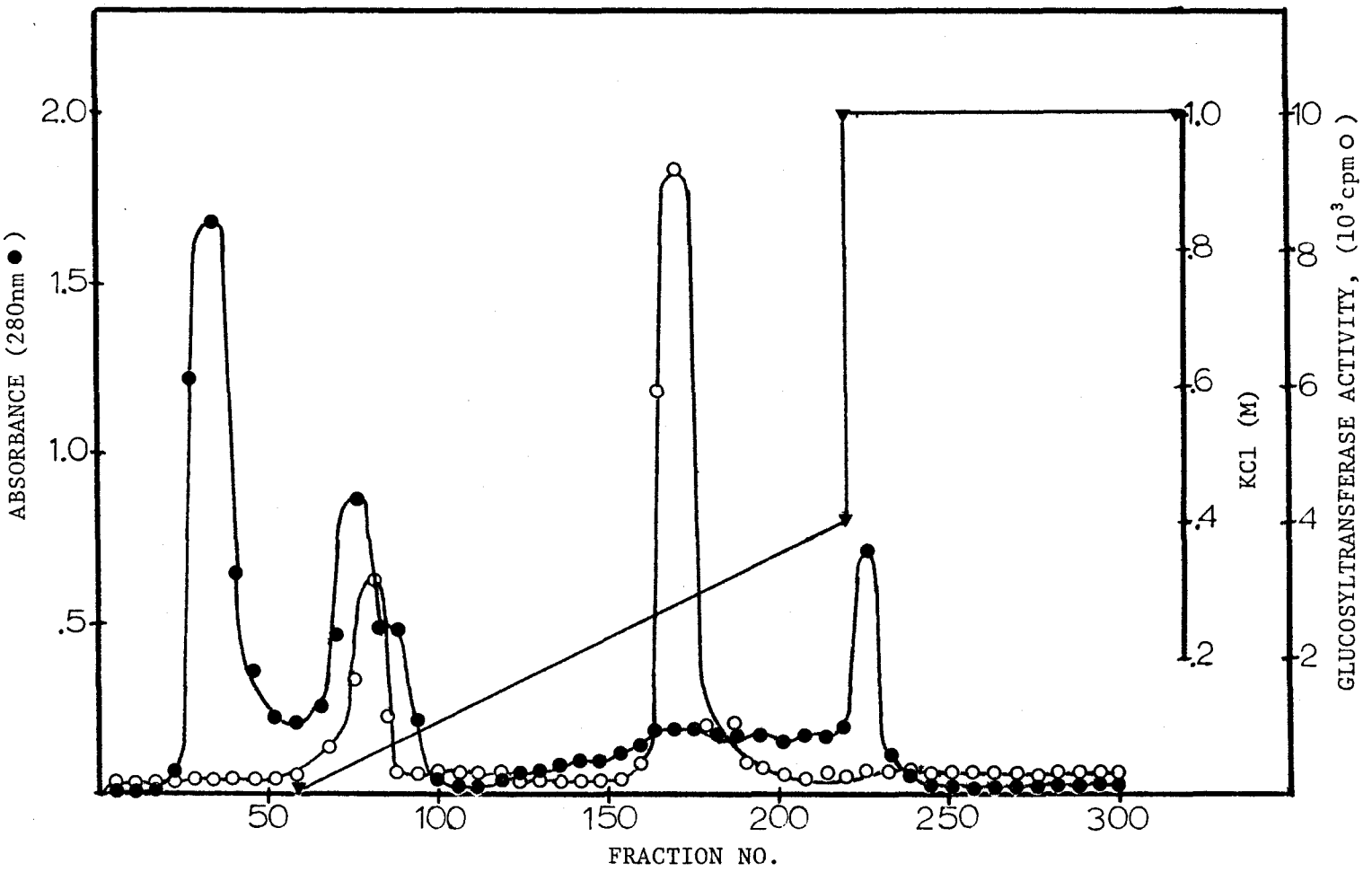

FIGURE 2. DEAE cellulose chromatography of ammonium sulfate precipitated and dialyzed TYG glucosyltransferase. Enzyme activity expressed as the counts (^{14}C) hexose polymerized in 20 min. under standard assay conditions.

broth supernatants. Glucosyltransferase in the TYF culture supernatant (Figure 3A) was stimulated approximately 3-fold in the presence of primer, over the unprimed reaction. Both reactions showed a linear increase in alcohol insoluble dextran product. The unprimed TYG culture supernatant (Figure 3B) exhibited less of a dependency on primer with only a one-fold stimulation of activity. The primed TYG reaction was linear, but the unprimed reaction had a much lower initial rate that increased gradually until 50 minutes whereupon the reaction proceeded at a faster rate than the primed reaction. The DEAE cellulose separated glucosyltransferase enzyme activities were also assayed for primer stimulation (Figure 4). Glucosyltransferase activity from the TYF preparation (Figure 4A) was extremely primer dependent as demonstrated by the 6-fold stimulation of activity. Both the primed and unprimed reaction rates were linear. The TYG Enzyme I (Figure 4B) exhibited only a slight increase (1.3-fold) in activity in the presence of added primer. TYG Enzyme II (Figure 4C) however, showed more dependence on primer, approximately 5-fold. These results were similar to the TYF Enzyme. Levansucrase was assayed for in all purified enzyme preparations (Figures 4A,B,C), but no activity could be detected in any of the preparations for the 60 minute assay incubation period.

Cariogenic streptococci produced both glucosyltransferase and dextranase during normal growth and plaque formation. In

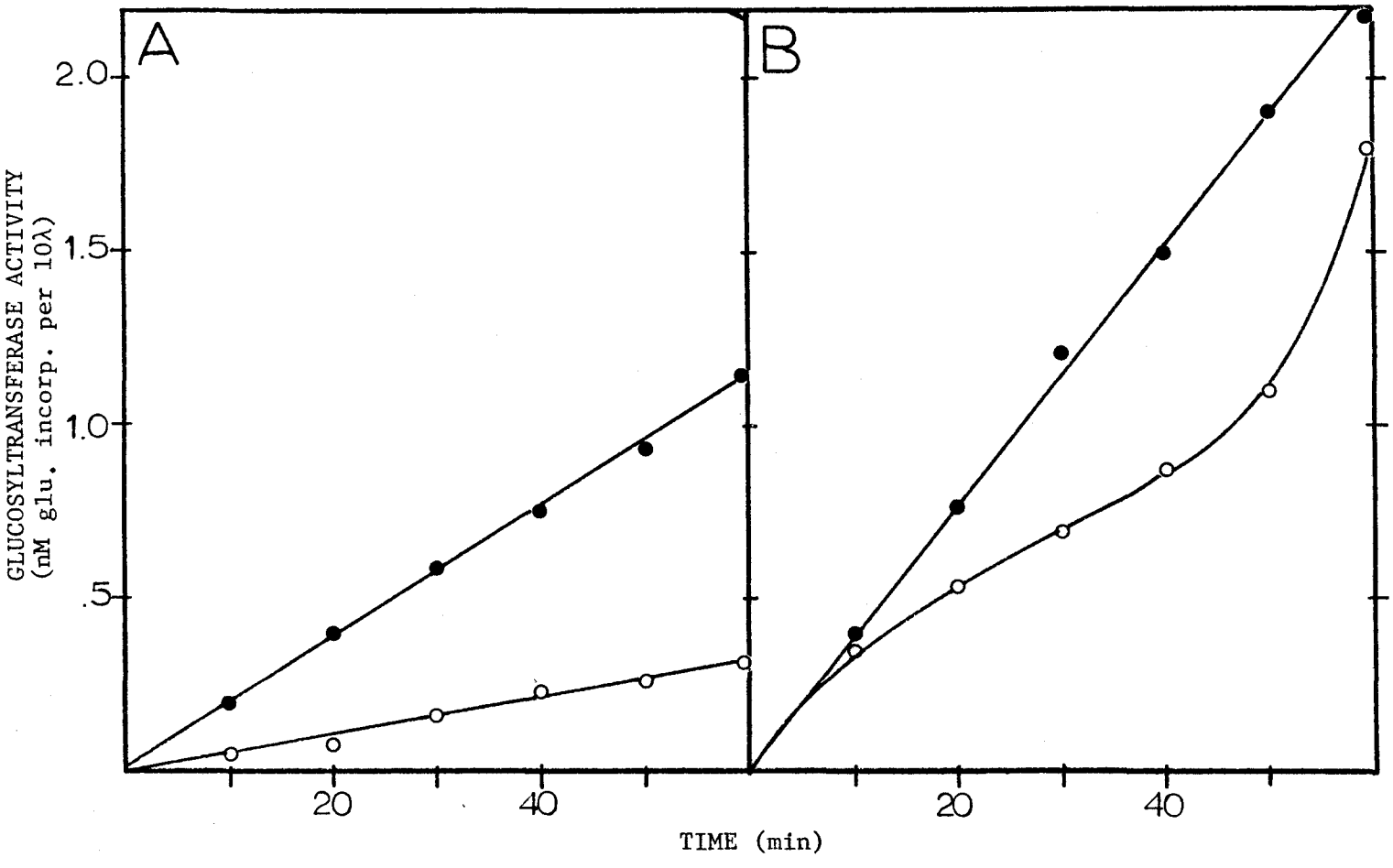

FIGURE 3. Effect of dextran T_{10} on glucosyltransferase activity from culture supernatents. Final protein conc. of culture supernatents in assay; (A) TYF=1.8mg per ml (B) TYG=.93 mg per ml. Symbols; (o) no T_{10} dextran, (●) $2.5X10^{-5}M$ T_{10} dextran.

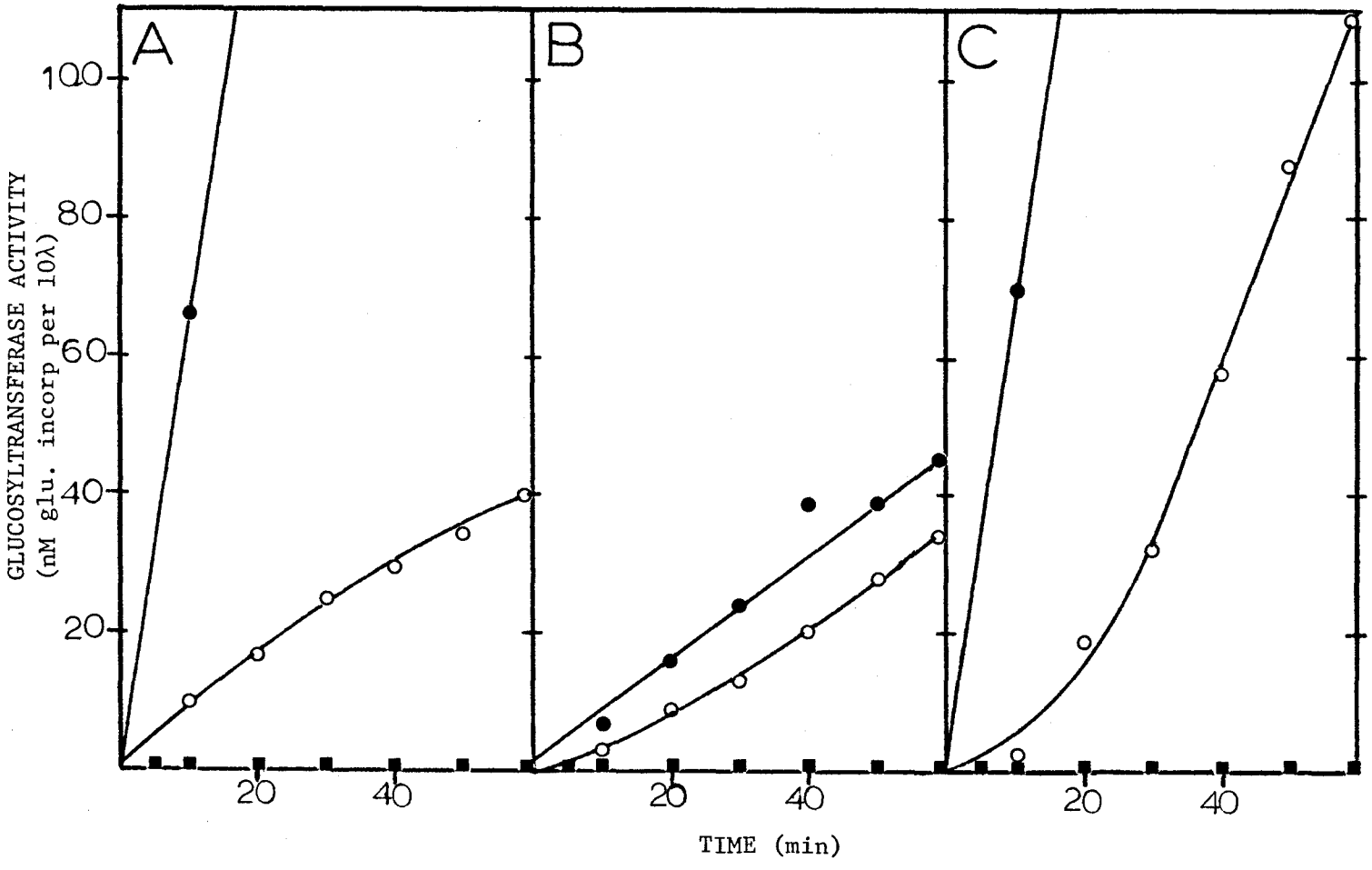

FIGURE 4. Effect of dextran T_{10} on DEAE cellulose glucosyltransferase activity. Final protein conc. of DEAE preparations in all assays; (A) TYF=.45 mg per ml, (B) TYG I = .03 mg per ml, (C) TYG II=.17 mg per ml. Symbols; (○) no T_{10} dextran (●) 2.5×10^{-5}M T_{10} dextran, (■) levansucrase activity.

order to study this enzyme relationship all glucosyltransferase preparations were assayed for alcohol insoluble dextran production in the presence of a commercial endohydrolytic dextranase preparation. Figure 5 shows the effect of dextranase on dextran synthesis by both primed and unprimed TYF culture supernatant glucosyltransferase. The unprimed TYF culture supernatant enzyme (Figure 5A) appeared to be much more susceptible to dextranase action over the primed TYF culture supernatant activity (Figure 5B). The unprimed TYF reaction leveled off immediately upon the addition of dextranase and did not show any change in the net synthesis of dextran for approximately 3 hours. The primed TYF reaction showed the same leveling off after the addition of dextranase but this lasted only 30 minutes, whereupon the enzyme resumed its normal rate of dextran synthesis. After one hour dextran synthesis again decreased to about half the normal rate. Comparing both reactions, the primed reaction showed a two-fold increase in glucosyltransferase activity over the unprimed reaction in the presence of dextranase. The TYG culture supernatant glucosyltransferase activity in the presence of dextranase is shown in Figure 6. The TYG reaction, although having more than a 4-fold increase in enzyme activity over the TYF reaction, still showed dextranase susceptibility in net polymer production. The comparative effect of dextranase was approximately equal for both enzyme preparations since the unprimed glu-

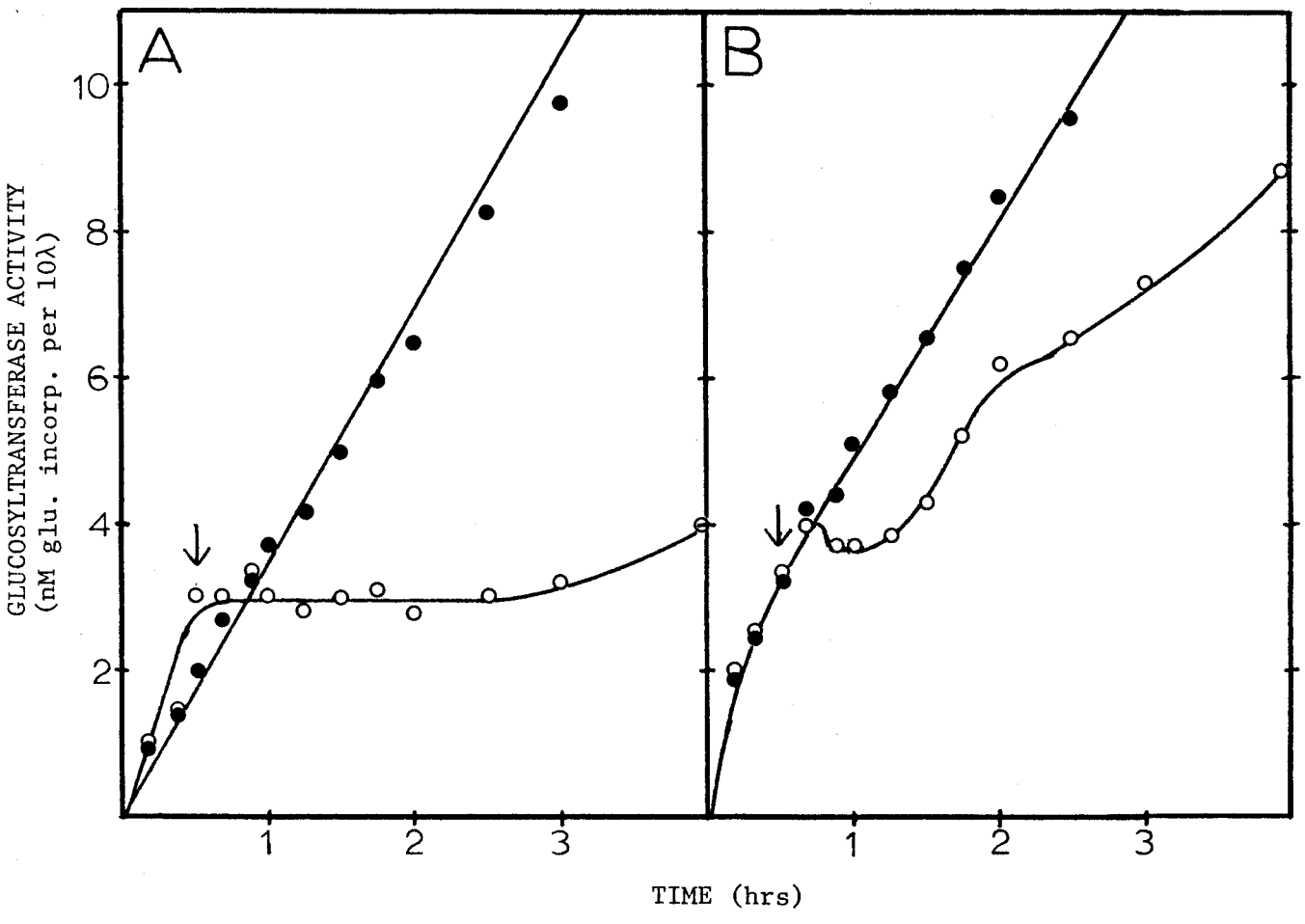

FIGURE 5. Effect of dextranase on TYF culture supernatent glucosyltransferase activity. TYF culture supernatent enzyme was assayed under normal assay conditions in the absence (A) and presence (B) of 2.5×10^{-5}M T_{10}dextran. Arrow indicates the addition of dextranase to the reaction mixture. Symbols; (●) no dextranase, (o) 6µg dextranase per ml.

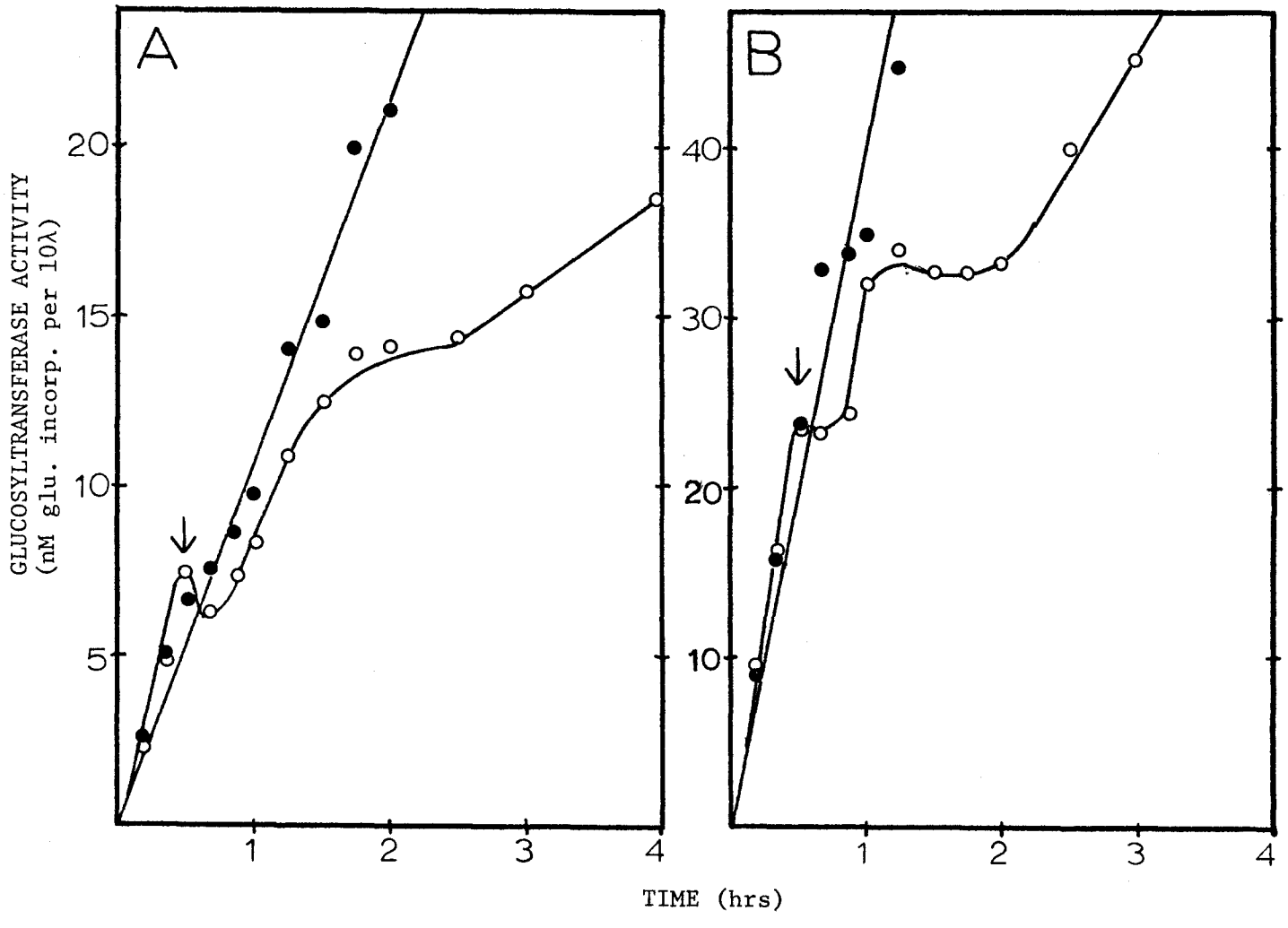

FIGURE 6. Effect of dextranase on TYG culture supernatent glucosyltransferase activity. TYG culture supernatent enzyme was assayed as in Fig. 5 in the absence (A), and presence (B) of T_{10} dextran. Arrow indicates the addition of dextranase. Symbols; (●) no dextranase, (○) 6μg dextranase per ml.

cosyltransferase activity was 3-fold less than the primed. Both reactions gave an initial decrease in polymer production, followed by an increase with the slight leveling off before resumption of dextran synthesis at a reduced rate. The primed TYG preparation exhibited this sequence of reactions more rapidily than the unprimed; however, both reactions gave similar results.

Similar assays were performed using partially purified enzyme preparations. Figure 7 illustrates the effect of dextranase on dextran production by TYF DEAE cellulose enzyme preparation. The unprimed TYF enzyme showed an initial decrease followed by little or no net synthesis of dextran. The primed TYF enzyme showed an initial drop in total dextran followed by increases and decreases in net polymer production. After 2.5 hours the reaction increased in rate approximately equal to the unprimed TYF enzyme without dextranase. The separated TYG glucosyltransferases were similarly tested as shown in Figures 8 and 9. The unprimed Enzyme I (Figure 8A) gave a decrease in polymer synthesis for 20 minutes after dextranase was added. This was followed by a somewhat normal rate of dextran synthesis with only one interruption after 75 minutes. Primed TYG Enzyme I (Figure 8B) also showed the fluctuation in polymer production, but it occurred after a longer time (1 hour) than in the other dextranase involved experiments (Figures 5, 6, 7). Also, unlike the unprimed, the primed reaction never reached the rate of the normal reaction

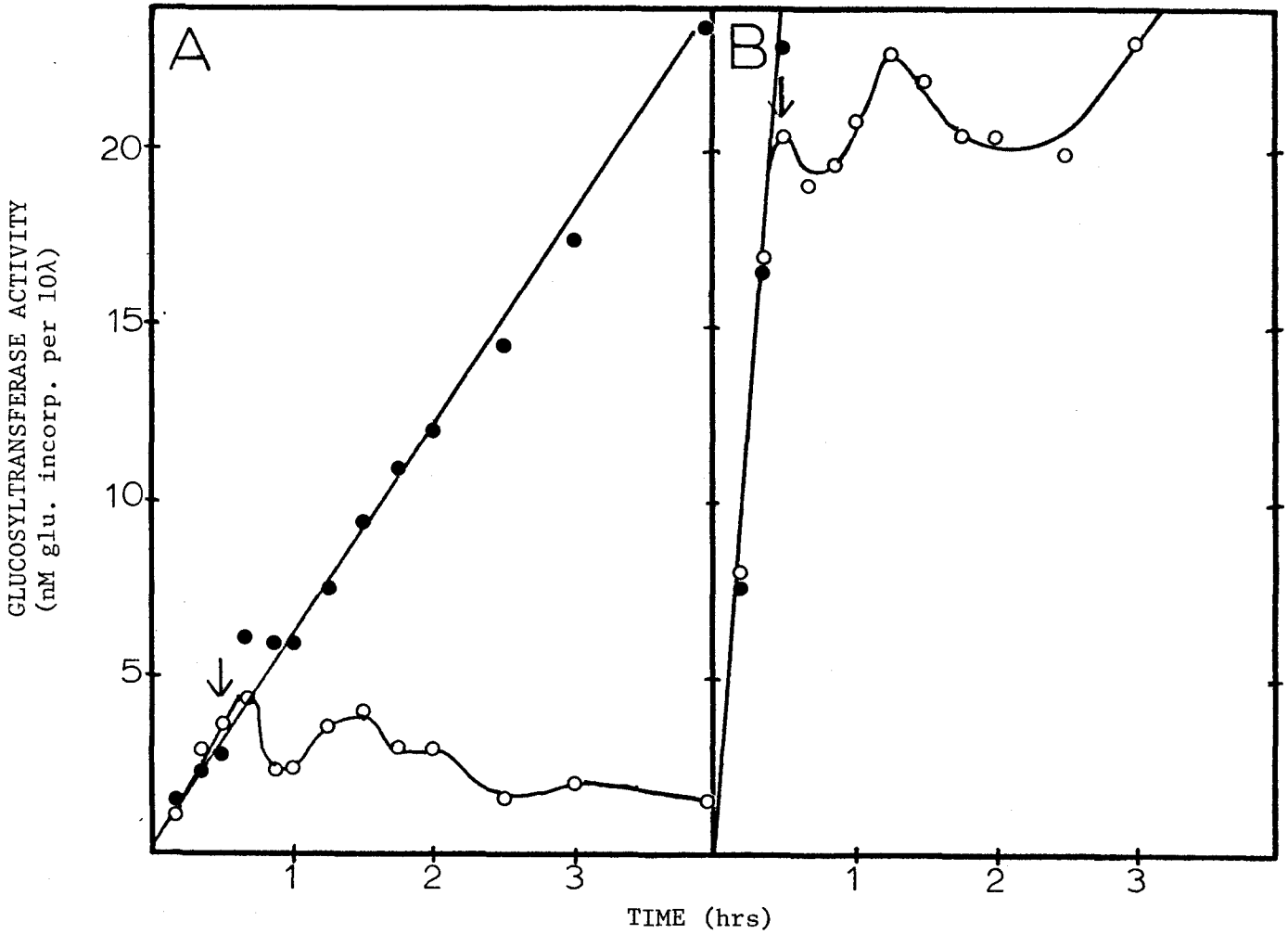

FIGURE 7. Effect of dextranase on TYF DEAE cellulose glucosyltransferase activity. TYF DEAE enzyme preparation assayed in the absence (A), and presence (B) of dextran (fig. 5). Arrow indicates the addition of dextranase. Symbols; (●) no dextranase, (○) 6μg dextranase per ml. TYF protein conc. as in Fig. 4.

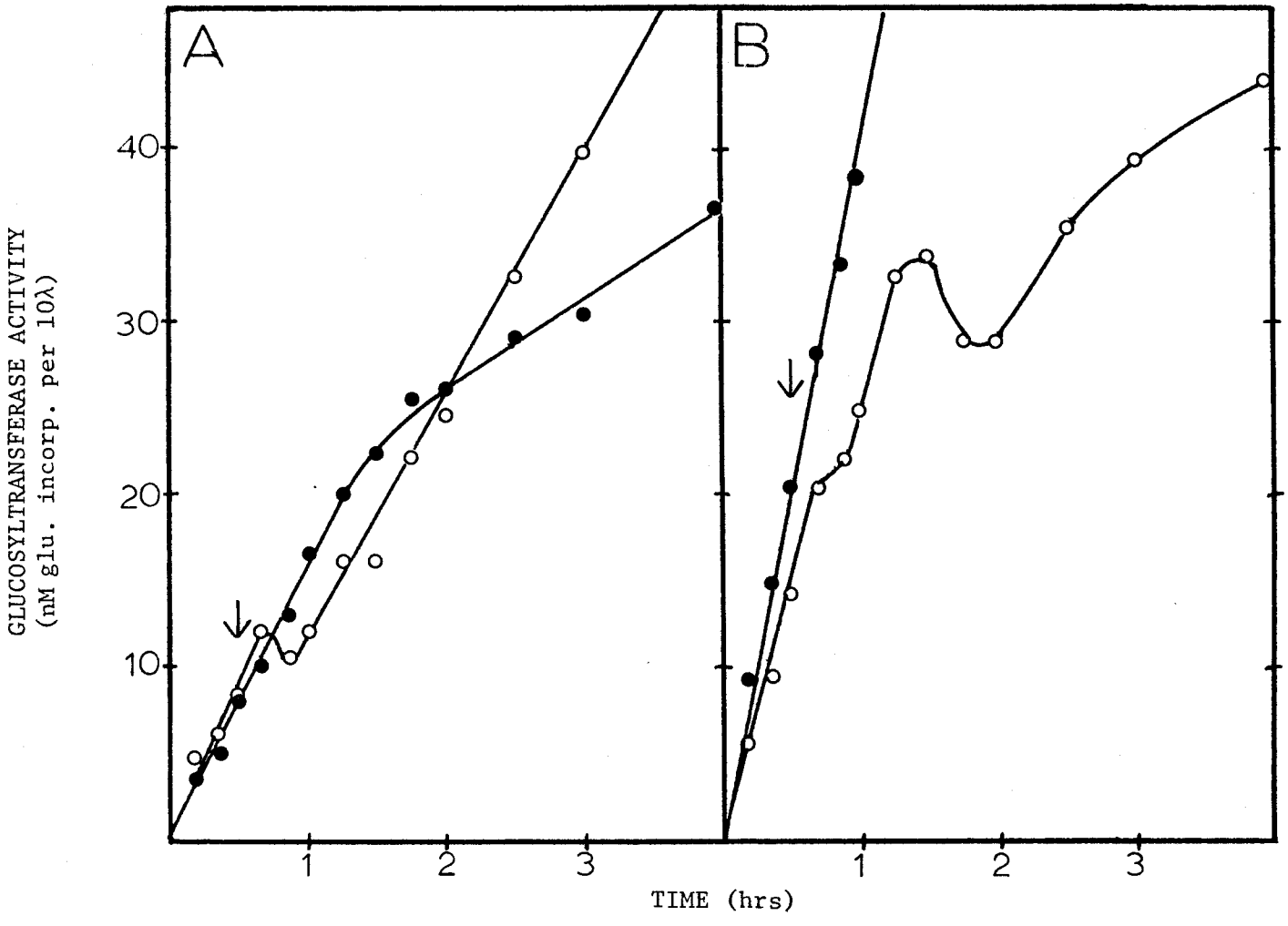

FIGURE 8. Effect of dextranase on TYG I DEAE cellulose glucosyltransferase preparation was assayed in the absence (A), and presence (B) of dextran (fig. 5). Arrow indicates the addition dextranase. Symbols; (●) no dextranase, (o) 6µg dextranase per ml. TYG I protein conc. as in Fig. 4.

without dextranase. TYG Enzyme II (Figure 9) exhibited a reaction with dextranase similar to that observed for the TYF glucosyltransferase. After an initial fluctuation, the unprimed reaction (Figure 9A) leveled off while the primed (Figure 9B) increased during the remainder of the assay time (4 hours).

Since dextranase altered net dextran production in both the primed and unprimed reactions, and this endohydrolase produces small isomaltodextrins, it was necessary to test the effect of small exogenous isomaltodextrins on dextran synthesis. Isomaltodextrins were prepared by acid hydrolysis of T_{10} dextran and separation of the isomaltodextrins mixture was performed by gel filtration on Sephadex G-25 (Figure 10). Isomaltodextrins were detected by measuring total hexose content of each fraction. The degree of polymerization (D.P.) of the fractions was determined by measuring the hexose/reducing ends in each fraction as described in Materials and Methods. (Table 3.) In Figure 10, the position and relative priming ability of the isomaltodextrins are shown using TYF Enzyme. The activity of the TYF enzyme as the average D.P. increases from 6 to 55, which is approximately the size of dextran T_{10} (10,000 M.W.). Enzyme activity with T_{10} and no primer is shown in the left portion of Figure 10. Isomaltodextrins with a D.P. of 12 were used as a source of primer in order to test their effect on dextranase action against alcohol insoluble dextran production by TYF enzyme (Figure 11).

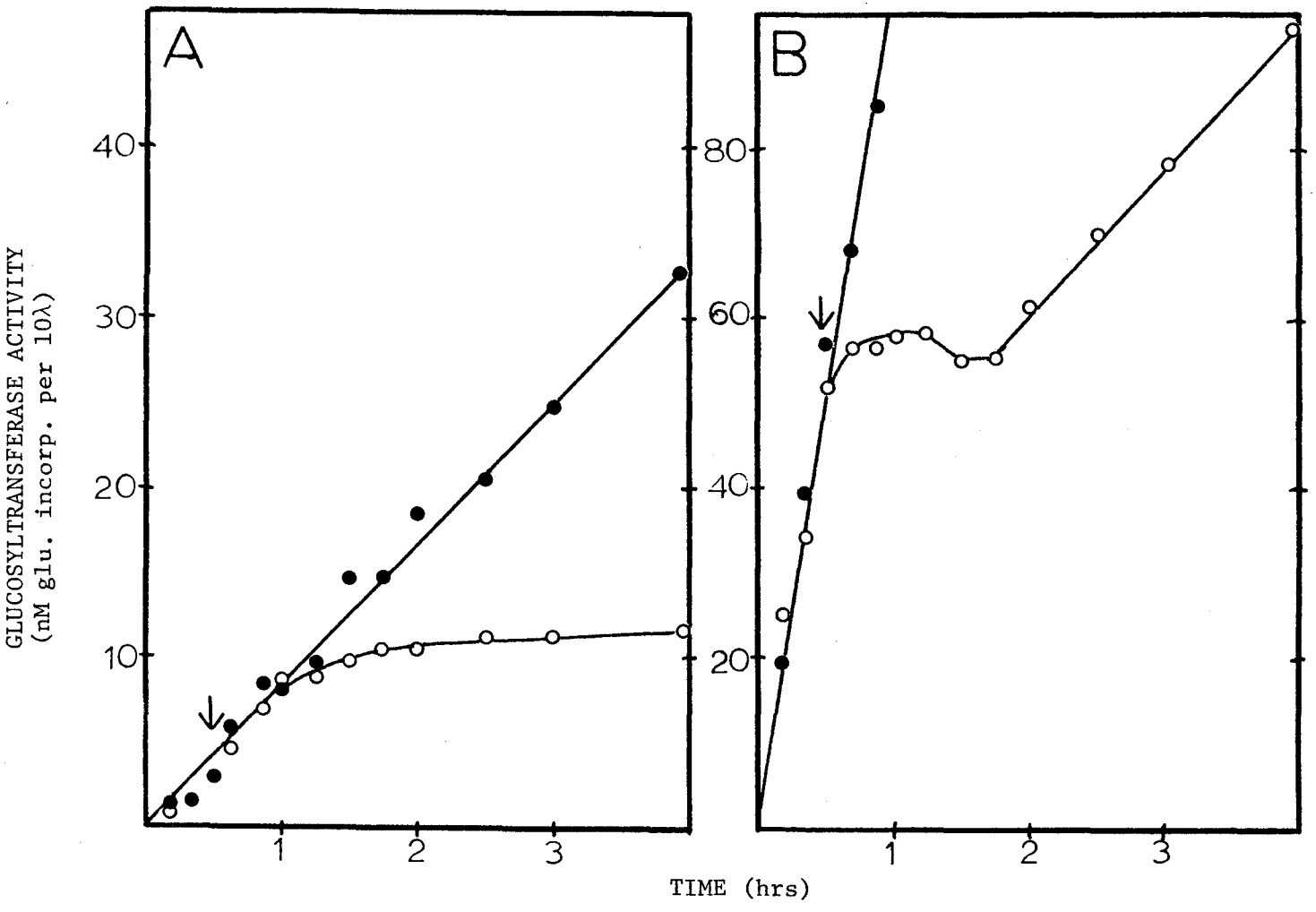

FIGURE 9. Effect of dextranase of TYG II DEAE cellulose glucosyltransferase activity. TYG II assayed in the absence (A), and presence (B) of dextran (fig. 5). Arrow indicates the addition of dextranase. Symbols; (●) no dextranase, (○) 6µg dextranase per ml. TYG II protein conc. as in Fig. 4.

Under both conditions of dextranase addition at time 0 and time 30, no inhibition of net polymer synthesis took place when compared to the reaction with no dextranase. So, water insoluble dextran product was not affected by dextranase in the presence of isomaltodextrins D.P. 12 as a source of primer. However, the characteristic fluctuation resulted.

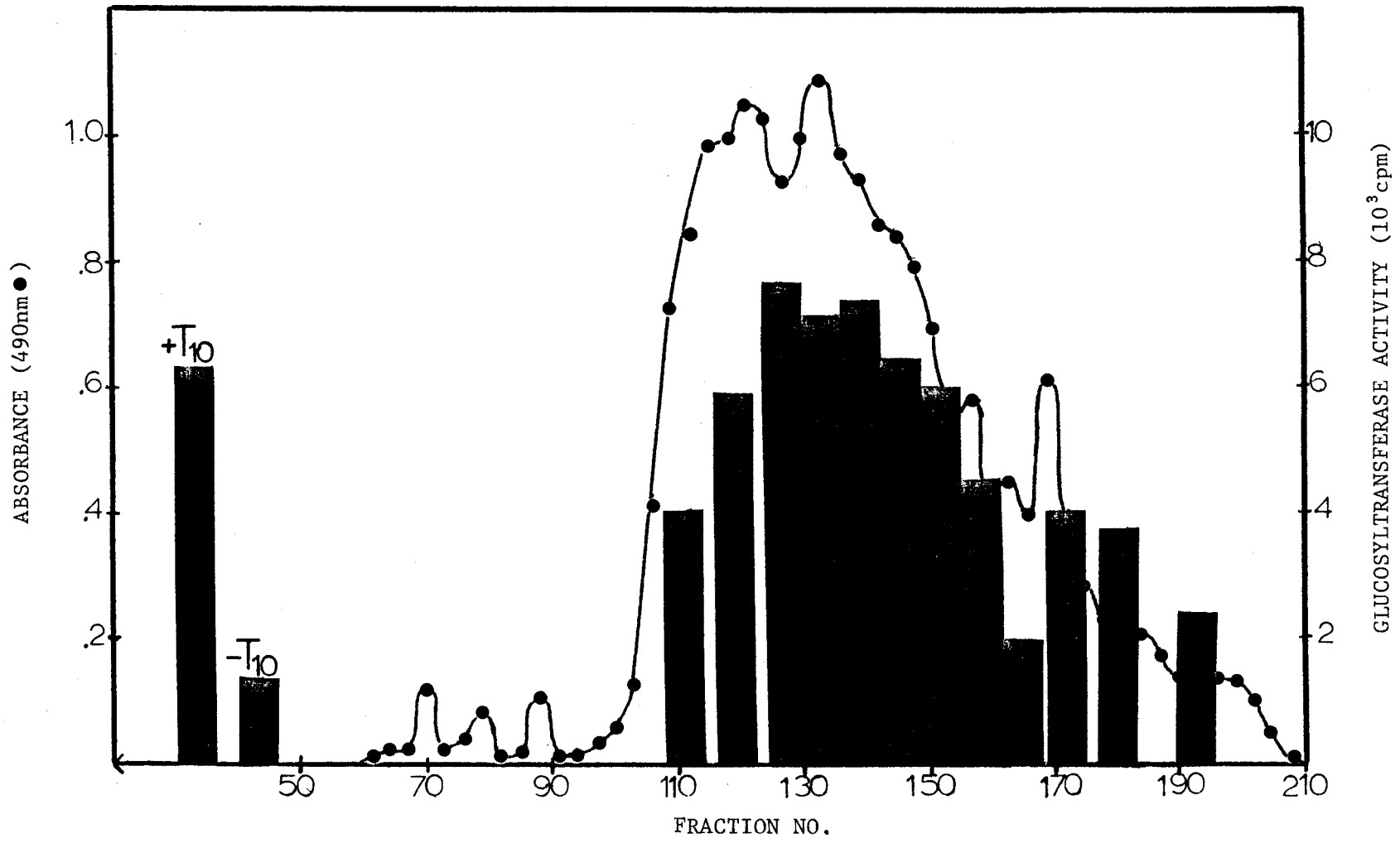

FIGURE 10. Sephadex G-25 gel filtration of T_{10} hydrolysate. Relative priming ability of fractions with similar D.P., after pooling, shown by bar graph. Assay using TYF DEAE enzyme described in M. and M. Enzyme activity in the absence and presence of 2.5×10^{-5}M T_{10} dextran is illustrated.

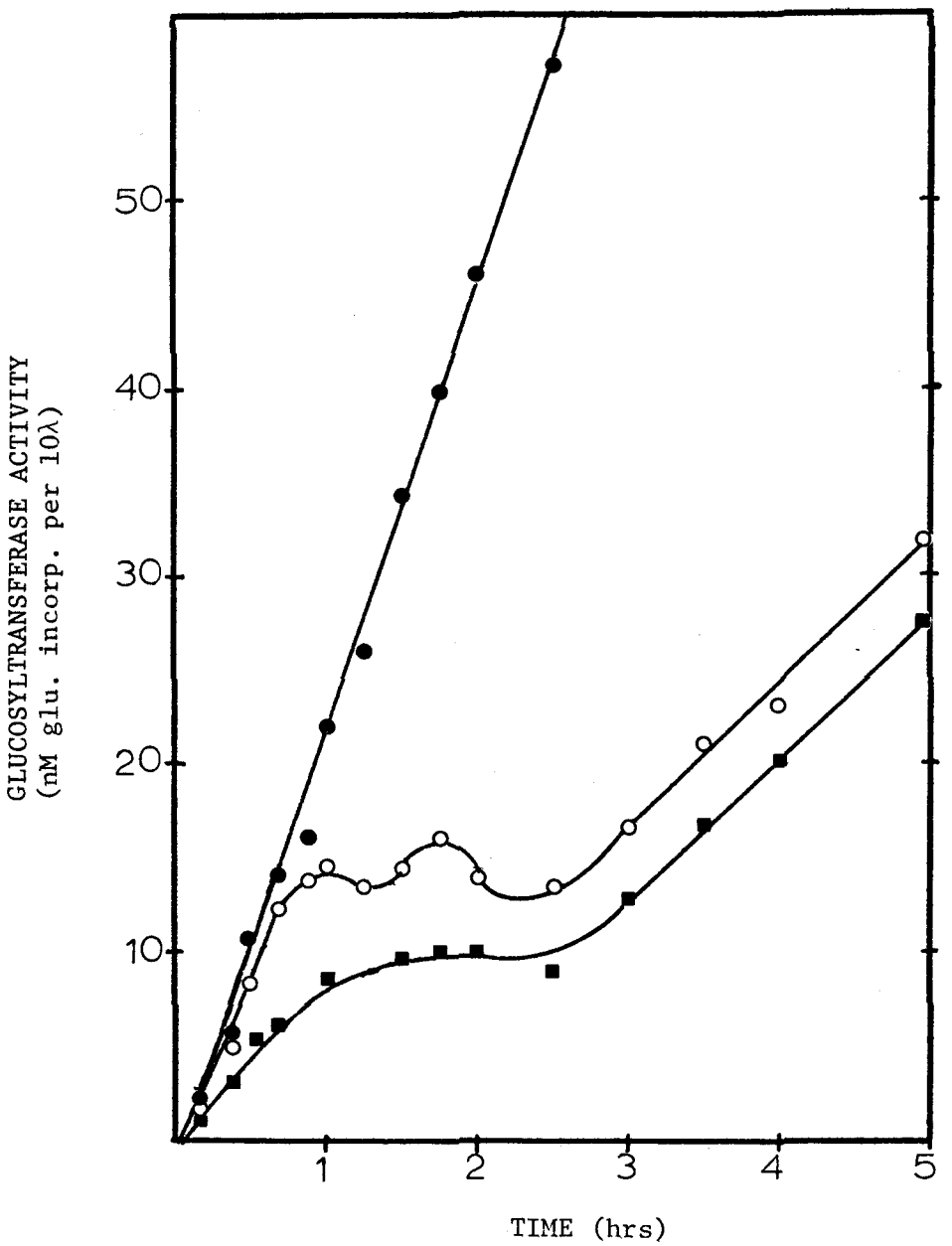

FIGURE 11. Effect of dextranase on isomaltodextrin primed TYF DEAE enzyme activity. Assay was performed as described in M. and M. using .025 mM of D.P. 12 isomaltodextrins as a source of primer. Symbols; (●) no dextranase, (○) 6µg dextranase per ml added at time 30 min, (■) 6µg dextranase per ml added at time 0 min.

CHAPTER V

DISCUSSION

The etiology of dental caries lies in the production of extracellular insoluble dextran polymers by the glucosyltransferase enzyme system of cariogenic Streptococcus mutans.[29,55,63] An ultraviolet mutant strain, S-19, of the parent strain 6715, was used in this study because of its capacity to produce elevated levels of the enzyme glucosyltransferase. It was shown that this organism produced an enzyme activity 3- to 6- fold higher than the parent strain enzyme.[77] Reports on the properties of glucosyltransferase from Streptococcus mutans have been variable depending on the culture media used, method of purification, etc. Two enzyme fractions have been reported,[10,33,76,68,62,78,35,36,] one being a monomer with a molecular weight of approximately 40,000 and producing water soluble polysaccharide while the other being an aggregate of the monomer and forming endogenous water insoluble polysaccharide.[36]

The present study reports differences in glucosyltransferase properties when the organism is grown under variable conditions. Using an enriched media supplemented with 0.5 M fructose, TYF, only one enzyme peak was resolved after DEAE cellulose chromatography (Figure 1). Using a chemically modified media substituting glucose for fructose, TYG, two peaks eluted (Figure 2). These results with TYG are similar to those reported by Germaine[35] who isolated two

glucosyltransferase enzymes with DEAE cellulose chromatography. It was concluded from that study, that one enzyme was in an aggregated high molecular weight form, while the other was a smaller molecular weight nonaggregated form. Investigations have also shown, that when Streptococcus mutans was grown in a defined media without the presence of contaminating sucrose, or in a media which was high in fructose concentration, aggregation could be reduced.[78] The TYF media in this study confirms this. Because of the high fructose concentration of TYF, only one enzyme was formed in the culture media due to competitive inhibition with sucrose by fructose. Therefore, endogenous dextran formation necessary for aggregation of the enzyme molecules could not take place. Table 1 shows this phenomenon by illustrating an increase in enzyme yield (activity) when fructose was removed from the enzyme preparation. In the present study, TYG Enzyme I produced marked precipitation of polysaccharides with the added sucrose, under assay conditions. TYF Enzyme and TYG Enzyme II however did not. These results are also corroborated by others.[35] It can be concluded therefore that TYG I is an aggregated form of the enzyme glucosyltransferase producing water insoluble polymer while TYG II and TYF Enzymes are nonaggregated or less aggregated forms producing predominantly water soluble polymers.

Under the appropriate conditions, glucosyltransferase is primer dependent, in that molecules of exogenous or endogenous product dextran can act as primer (usually requiring a minimum

size of 8 glucose units), increasing enzyme activity.[10, 34, 36] In the absence of primer, the reaction proceeds until enough endogenous dextran is built up to self prime the reactions resulting in an autocatalytic effect. Germaine[36] showed that primer dextran could act by binding more than one glucosyltransferase molecule resulting in the formation of branch points on the linear dextran chain and producing aggregates of an enzyme-dextran complex. In the absence of dextran the high molecular weight aggregates did not form until the reaction proceeded sufficiently to produce small dextrans, of at least 8 glucose residues long resulting in autocatalysis. The results of the present study confirm these findings. The non-aggregated TYF culture supernatant TYF Enzyme and TYG II Enzyme preparations (Figures 3A, 4A, 4C) showed this primer dependency with an increase in enzyme activity of 2-, 8-, and 6.5-fold respectively. The TYG culture supernatant, having both enzyme forms showed a limited primer stimulation while the TYG I Enzyme showed no primer stimulation since these preparations were already in the aggregated form and no added exogenous dextran could stimulate the reaction further.

Plaque is formed as a result of extracellular polysaccharide production, i.e. insoluble α (1,3) linked, α (1,6) branched dextran by cariogenic strains of Streptococcus mutans.[66, 67, 72, 73, 79] It has also been found that many of the organisms which comprise this ecological nitch of the oral cavity produce enzymes which have the ability to hydrolyze dextran molecule. Among these are species of

Actinomyces, Bacteroides and specifically strains of Streptococcus mitis and Streptococcus mutans.[80,86,93,87] Guggenheim[44] isolated an α (1,6) endohydrolytic dextranase from Streptococcus mutans OMZ 176. Because of the seemingly inconsistent phenomenon of S. mutans forming and possessing the potential for the breakdown of dextran, the action of dextranase on streptococcal dextran formation was studied. It has been shown that in early dextran synthesis, glucosyltransferase forms α (1,6) linked isomaltodextrins, by attaching the glucosyl moiety from sucrose to an acceptor molecule at the non-reducing terminus.[92] Utilizing this information, Schachtele[80] showed that an exohydrolytic dextranase completely blocked glucosyltransferase's water insoluble glucan production and its adhesion by inhibition of the initial α (1,6) linkages in dextran formation. Ebisu[25] showed that the structure of an insoluble dextran produced by S. mutans consisted of a linear α (1,3) linked glucan, with α (1,6) branches resulting in the insolubility and adhesive qualities of the polysaccharide. When formed in the presence of an endohydrolytic dextranase, this glucan still retained the α (1,3) backbone but no α (1,6) side chains formed. This structure resulted in an insoluble glucan with no adhesive qualities. This endohydrolytic dextranase was also shown by Stuart[87] to hydrolyze α (1,6) linked isomaltodextrins with a size no smaller than 10 glucose units long. These results suggest that an endohydrolytic dextranase is capable of degrading α (1,6) linked glucan side chains

10 glucose units long or longer, which give adherence, but not α (1,3) backbone linkages which are responsible for the glucan's water insolubility. The results of the present study clearly indicate this point. Since the nonprimed TYF culture supernatant, DEAE TYF and TYG II glucosyltransferases (Figures 5A, 7A, 9A respectively) are not aggregated, the α (1,6) soluble product from these preparations would be inhibited resulting in no autocatalytic effect whatsoever. When these same preparations are primed however, (Figures 5B, 7B, 9B respectively) the enzymes aggregate and form insoluble polysaccharide which is less effected by the action of dextranase. Any net loss of total dextran after the addition of dextranase was probably due to the limitation of this filter paper assay which only retained isomaltodextrins greater than D.P. of 6 or 7 on the filters. The TYG culture supernatant and TYG Enzyme I contain aggregated forms of the glucosyltransferase enzyme. Therefore, in the absence of primer, the TYG culture supernatant and TYG Enzyme I (Figures 6A and 8A) showed no inhibition of net dextran synthesis. On the contrary, both showed a positive net polymer production with the TYG I Enzyme giving higher polymer production in the presence of dextranase than in its absence. Supposedly this occurs because the isomaltodextrin products of dextranase are incorporated into an aggregate which produces insoluble dextrans; these insoluble dextrans are less susceptible to dextranase action because of their α (1,3) linkages. Therefore the "autocatalytic

effect" is not interferred with by dextranase because aggregated enzyme which would eventually form from even a monomeric or non-aggregated enzyme cannot be hydrolyzed, only free dextran longer than 10 units long. A dextran molecule 8 units long however can prime a reaction and form aggregate. These results are reinforced by Germaine,[35] who isolated α- and β- glucosyltransferase enzymes producing water insoluble and water soluble polysaccharides respectively. The α preparation contained a dextranase activity which could modify the glucan produced by β into water insoluble glucan.

In all dextranase assays, there appeared a fluctuation in the net polymer production by glucosyltransferase. The reason for this may be the dependence of dextranase on a minimum size α (1,6) linked side chain (10 units) for hydrolytic activity. In Figure 7B, for example, an initial decrease in net polymer synthesis took place due to the availability of the side chains and hydrolysis of them by dextranase resulting in the pieces being washed off the filter paper. After one hour, net synthesis resumed apparently due to the reincorporation of these small pieces into the aggregate glucosyltransferase. Glucosyltransferase molecules could then lengthen these side chains after a period of time due to the autocatalytic effect and now make them available to dextranase again. However, when the cycle repeats itself, less side chains are available than before because of an

increase in aggregation, and insolubility of the polymer. To test the phenomenon isomaltodextrins with varying D.P. were collected from the gel filtration of T_{10} hydrolysate (Figure 10). Priming ability of these fractions were tested and fractions with a D.P. of approximately 12 or greater had the greatest effect in increasing enzyme activity of TYF enzyme. These results are corroborated by Germaine.[34] Since isomaltodextrins with a D.P. of 12 seemed to be the smallest size which could still prime the reaction, and also be of sufficient size to be hydrolyzed by dextranase, an attempt was made to prime TYF Enzyme in the presence of dextranase (Figure 11). Net polymer production was not inhibited by dextranase when primed by the isomaltodextrin. Glucosyltransferase activity fluctuated as before, however, due to the presence of susceptible primer and side chains. After a period of time equilibrium is reached as before with a positive net polymer production due to aggregate formation and less susceptible α (1,6) linked side chains. This fluctuation was not as apparent when dextranase was added at time 0 minutes than when dextranase was added at time 30 minutes. This was due to the fact that dextranase was present in the reaction mix initially and utilized to form the insoluble product more gradually, with the same result of a positive net polymer production not susceptible to dextranase activity.

SUMMARY AND CONCLUSIONS

The effect of dextranase in decreasing plaque formation has not been shown by these data. The interrelationships between the multicomponent glucosyltransferase, endogenous dextran, sucrose, and dextranase is too complex to expect dextranase to be able to remove plaque by disrupting the plaque matrix: i.e. water-insoluble dextran. These data show just the opposite, in that dextranase acts to break up water-soluble dextran produced by one component of the glucosyltransferase scheme providing primer dextran to the other component of the glucosyltransferase enzyme system. This primer then functions to form aggregates which are impervious to dextranase activity, and the aggregates form insoluble polymer, agglutinating the bacterial cell and adhering to the tooth surface.

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science in Oral Biology.

April, 1978
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