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Evaluation of the Essentiality of Dextran in the Dental Caries Process

William Redman Grigsby

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Evaluation of the Essentiality of Dextran in the Dental Caries Process

by

William Redman Grigsby

B.A., Dartmouth College, 1956
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Thesis submitted in partial fulfillment of the requirement for the Degree of Doctor of Philosophy in the Department of Biochemistry at the Medical College of Virginia Health Sciences Division Virginia Commonwealth University Richmond, Virginia

June 1970
This thesis by William Redman Grigsby
is accepted in its present form as satisfying the thesis requirement
for the degree of Doctor of Philosophy.
CURRICULUM VITAE
PREFACE

It is my desire that this thesis will have served two purposes:

1. Claude Bernard has written that "nobody reached truly illuminating and fruitful generalizations concerning vital phenomena who has not himself experimented and tilled in hospital, operating theater, and laboratory the fetid and palpitating soil of life." This thesis documents partial fulfillment of the experimentation and tilling requirements Bernard has set forth.

2. Hopefully this thesis serves as a downpayment on the debt I owe the American Taxpayer (N. I. D. R. Dental Training Grant #ST01-DE 00008-1; and N. I. H. General Research Support Grant #FR-05345-06) and as a manifestation of my continuing dedication to contributing positively to the improvement of human, oral health.
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Chapter 1

INTRODUCTION

Dental caries or tooth decay has been defined in the 24th edition (1965) of Dorland's Medical Dictionary as "a disease of the calcified tissues of the teeth resulting from the action of microorganisms on carbohydrates, characterized by a decalcification of the inorganic portions of the tooth and accompanied or followed by disintegration of the organic portion." Dental caries is more ancient than man and this disorder has probably always plagued him (Thoma, 1954 and Burnett and Scherr, 1968). Tooth decay is today the most prevalent chronic disease affecting the modern human race (Shafer, Hine, and Levy, 1966). Ninety-eight percent of all Americans are affected at some time during their lives by tooth decay (Public Health Publication No. 1483, 1966). In 1958 the American public spent approximately $800 million for fillings to combat dental caries (Shafer, Hine, and Levy, 1966). This enormous health and economic problem has stimulated much dental caries research which has resulted in a vast literature on this subject. Therefore, an attempt has been made to select critically those papers which bear directly on the problem treated in this thesis.

Black (1886) isolated a "gelatine forming oral cocci" which "gelatinized so perfectly" the culture tube contents (peptonized broth with 2% sugar) that the tube could be inverted without spilling. Black wrote that "this will give some idea of what the coccus may do in gumming up the teeth, . . . ." He also perceived that the material
he referred to as gelatine "does not melt or soften at high tempera-
ture, and in many respects differs from what we know as gelatine."

Miller (1890) concluded from his studies of bacterial fermentation
that "dental decay is a chemico-parasitical process consisting of two
distinctly marked stages: decalcification, or softening of the tis-
sues, and dissolution of the softened residue." He reasoned that "it
is not difficult to determine the source of the acids which effect the
decalciﬁcation. They are derived chiefly from particles of amylaceous
and saccharine substances which lodge in the retaining-centers and
there undergo fermentation."

Williams (1897) produced the first visual evidence for bacterial
colonization of teeth with photomicrographs. Black (1898) designated
the bacterial accumulation covering the tooth surface shown in
Williams' pictures as a "gelatinous microbic plaque".

Belding and Belding (1940) observed that some oral streptococci
formed large mucoid colonies on blood-sucrose agar. Niven, Smiley,
and Sherman (1941 a and b) first showed that the polysaccharide asso-
ciated with mucoid colonies of oral streptococci was levan and possi-
bly dextran. Stephan (1953) wrote: "It should be pointed out that the
'gelatinous substance' which Black described was probably a polysac-
charide, formed by various streptococci. . . ."

A milestone in dental caries investigation was the use of germ-
free animals (Orland et al., 1954). These workers demonstrated that
none of 22 germfree Lobund white rats experienced dental caries

Plaque or dental plaque is interpreted and used (in this thesis)
to mean the non-calcified, microbial accumulation that adheres tena-
ciously to teeth.
although these germfree animals ingested the same diets that allowed carious lesions to develop in 38 of 39 conventional microbe-bearing Lobund white rats. This result suggested some agent or agents in the conventional rats' microbial flora was responsible for their dental caries. Later, (Orland et al., 1955 and Orland, 1964) a single microbial agent (an enterococcus isolated from a carious rat molar) was shown capable of inducing dental caries in the molars of gnotobiotic Lobund white rats. The lesions experienced by the conventional microbe-bearing animals and the monoinfected gnotobiotic animals were visually identical.

Snyder et al., (1955) examined polysaccharides synthesized by sucrose-grown bacteria isolated from human saliva. They found large numbers of levan-forming streptococci but concluded that "because of the water soluble nature of the mucinous polysaccharides (levan), their participation in the formation of dental mucinous plaques was believed unlikely." Dextran-forming streptococci of the human saliva were also considered as potential plaque formers but these authors wrote: "... the third, dextran-forming streptococci, occurs so rarely as to have no apparent significance in the overall process."

Fitzgerald, Jordan, and Stanley (1960) substantiated and extended the earlier gnotobiotic studies by using a different diet and a different microbial agent to induce carious lesions in the molars of gnotobiotic Lobund white rats. They utilized a micro-aerophilic, alpha-hemolytic streptococcus (later designated Streptococcus FA1) as a monoinfecting agent to produce tooth decay in the molars of sucrose-fed gnotobiotic rats.

Fitzgerald and Keyes (1960) showed that conventional, microbe-
bearing albino hamsters were resistant to dental caries on a diet containing 59% confectioner's sugar, 27% skim milk powder, 6% wheat flour, 3% alfalfa powder, 2% whole liver powder, 2% liver concentrate, and 1% Wesson salt mixture. The albino hamsters were inoculated with a variety of pure bacterial strains as single agents in addition to their normal flora. Six strains of lactobacilli isolated from the hamster oral cavity and six strains of diphtheroids isolated from a carious hamster molar were used as single agents to complement the normal bacterial flora. None of these organisms induced dental caries or formed dental plaque in the sucrose-fed, conventional hamsters. **Streptococcus FA1** and six strains of streptococci (4 oral and 2 fecal) isolated from caries inactive hamsters also failed to induce tooth decay when introduced as single agents in the sucrose-fed, conventional albino hamsters. However, each of five streptococcal strains isolated from the same carious hamster molar as the above diphtheroids induced dental caries to varying degrees in the molars of the albino hamsters. This experiment showed that a high sugar diet and acidogenic bacteria did not necessarily result in tooth decay. Dental caries development depended upon the presence of specific bacteria.

The concept that not all oral bacteria caused tooth decay was verified in the gnotobiotic system. Fitzgerald (1963) wrote that "we have now tested a pigmented heterofermentative lactobacillus, *Lactobacillus fermenti*, a homofermentative *L. acidophilus*, a *Streptococcus lactis*, and a proteolytic *S. faecalis* var. *zymogenes*, all of rat origin, as monoinfections in germfree animals, and have found them unable to induce caries, even though the animals were maintained on a cariogenic diet."
Jordan (1965) compared acid production from glucose by Streptococcus FA1, cariogenic in the rat, and Streptococcus JR8LG, non-cariogenic in the rat. He concluded that "the capabilities for glucose fermentation appear to be the same for both strains, at least under these conditions, and that their differences in caries activity must be explained on some other basis." Jordan also wrote that "this, of course, does not negate the importance of fermentation acids but it does indicate that acid production per se cannot be the sole determinant of cariogenicity." Drucker and Melville (1968) have corroborated and elaborated Jordan's work by concluding that "there is no significant difference between cariogenic and non-cariogenic streptococci as regards either the amount or the type of fermentation acids produced."

Jordan (1965) speculated that "seemingly peripheral factors such as ease of implantation, growth in the oral cavity and colonization on the tooth can be as important as the actual mechanism of tooth destruction in understanding the pathogenicity of these agents." This was a continuation of an earlier idea (Keyes and Jordan, 1963) which these authors expressed by writing: "If some of the bacteria (parasites) have a cariogenic potential, and if the food substrates are of the proper quality and quantity, colonization and plaque formation occur on certain surfaces of the teeth." They also noted that "not all acidogenic organisms will colonize and produce plaques on enamel surfaces." Finally they observed that "the plaque which so rapidly forms in either naturally infected or inoculated animals appears to be the product of an interaction between bacteria and diet." This observation was consistent with the work of Wood (1964) and McDougall (1964) who isolated levan from human plaque material. They concluded that the
levan was synthesized from sucrose by oral streptococci. Krasse (1965a and b) added support for this position when he showed that dietary sucrose compared to dietary glucose facilitated the implantation of cariogenic streptococci in the hamster oral cavity. The hamsters on the glucose-containing diet "did not show any gross plaque formation" but the hamsters on the sucrose-containing diet "developed heavy plaques."

Jordan and Keyes (1966) examined the ability of cariogenic and non-cariogenic streptococci to produce plaques. They developed an in vitro system which required salivary mucin and sucrose in which cariogenic streptococci formed adherent, visible plaques on stainless steel wires, glass electrodes, and extracted, human teeth. The non-cariogenic streptococci tested did not produce plaques under the same conditions. These authors wrote: "Plaque formation by the active strains in the in vitro model described provides a working hypothesis to explain the relationship of caries-conducive bacteria to carious lesions which occur on the smooth surfaces of enamel. The ability of these specific strains to colonize and form plaque on the tooth surface in the presence of sucrose and mucin would give them an advantage over the non-plaque-forming strains in the initiation of carious lesions."

Gibbons et al., (1966) suggested that "capsule formation may be important in the caries process." These investigators showed that seven strains of cariogenic streptococci produced significantly more "extracellular capsular" material than did three non-cariogenic strains of streptococci when all were grown on sucrose medium.

Wood and Critchley (1966) found that Streptococcus FA1 synthesized extracellular levan and dextran from sucrose. Wood (1967b) reported
the isolation of an extracellular, dextranucrase enzyme from glucose-grown Strep. FA1. Donahue, Kestenbaum, and King (1966) discovered that the cariogenic streptococci which they tested produced dextran-like polysaccharide from sucrose. The non-cariogenic strains examined did not synthesize a dextran-like polysaccharide under the same conditions. Gibbons and Banghart (1967) demonstrated that a number of cariogenic streptococci produced extracellular dextran from sucrose. This polysaccharide, dextran, formed an insoluble precipitate with whole, cleared saliva and was tenaciously bound to powdered hydroxyapatite from teeth. The dextran was resistant to attack by plaque or salivary bacteria. However, plaque and salivary bacteria readily hydrolyzed levan which agreed with Wood's (1964) finding. Gibbons and Banghart (1967) also noted that cariogenic Lactobacillus acidophilus 108TR produced a substance from sucrose immunologically similar to dextran. Dahlquist et al., (1967) determined that two strains of streptococci (Ingbritt and Mats), isolated from humans and capable of inducing carious lesions in albino hamsters, synthesized extracellular glucan and fructan when grown on sucrose-containing medium. Guggenheim and Schroeder (1967) characterized water insoluble dextrans produced by two strains of sucrose-grown streptococci (OMZ 61 from carious rat molar and OMZ 176 from human plaque).

Gibbons and Banghart (1967) showed that pooled, human dental plaque contained approximately 2 percent of the total dry weight as material antigenically identical to dextran. Critchley et al., (1967) concluded that human plaque contained extracellular polysaccharides of the levan and dextran type and that plaque bacteria could synthesize these polysaccharides in vitro and in vivo from sucrose. Wood (1967a)
calculated that extracellular polysaccharides made up 9 percent (standard deviation 3.5 percent) of the dry weight of human plaque and these polysaccharides were levan and dextran.

Gibbons and Banghart (1967) and Guggenheim and Schroeder (1967) employed dextranase preparations to characterize the dextrans they studied. Gibbons and Banghart found dextranase from *Penicillium funiculosum* NRRL 1768 degraded dextran from *Leuconostoc mesenteroides*. This dextranase preparation also hydrolyzed dextran from *Streptococcus GS-5*, an organism which had been isolated from a human and was found cariogenic for gnotobiotic rats. Fitzgerald, Spinell, and Stoudt (1968) degraded *in vitro* plaques formed on wires by cariogenic streptococcal strains, FA1 (rat source), SL-1 (human source), and E-49 (hamster source), with a dextranase preparation from *Penicillium funiculosum* NRRL 1768. Fitzgerald, Keyes, Stoudt, and Spinell (1968) fed dextranase to hamsters inoculated with *Streptococcus E-49*. The hamsters receiving dietary dextranase experienced reduced plaque formation and dental caries as compared to infected, non dextranase-fed, control hamsters. They concluded that their findings were consistent "with the concept that dextran production by oral microorganisms is a key requisite for plaque formation and caries induction in the hamster. Furthermore, the results provide an explanation for the fact that sucrose is a more caries-conducive dietary substrate in the hamster than other carbohydrates, for sucrose is the prime substrate for dextran production by the cariogenic streptococci that were used in the present study." They also reported that "it seems justifiable to conclude that the anticaries effects noted with dextranase resulted primarily because the dextranase prevented the cariogenic streptococci
present in the oral environment from colonizing on the surfaces of the teeth."

The dextranase studies (Fitzgerald, Spinell, and Stoudt, 1968 and Fitzgerald, Keyes, Stoudt, and Spinell, 1968) and the observations that levan is soluble and readily metabolized (Snyder et al., 1955, McDougall, 1964, Wood, 1964, Gibbons and Banghart, 1967, DaCosta and Gibbons, 1968, and VanHoute and Jansen, 1968) by plaque bacteria have suggested that dextran is the bacterial metabolite responsible for tooth colonization and therefore plaque formation (Burnett and Scherp, 1968). Therefore the hypothesis is that dextran is a requisite for dental caries. This position was stated by Gibbons and Banghart (1967) when they wrote:

The concept emerging from the present and previous studies is that the formation of extracellular dextran, particularly from sucrose, by cariogenic bacteria appears to enable these organisms to form plaque which is necessary for the production of dental caries. In contrast, non-cariogenic bacteria are unable to synthesize significant quantities of dextran and therefore cannot form plaque. Thus synthesis of certain extracellular polysaccharides would appear to be one of several characteristics required by a bacterium in order to be cariogenic.

Accepting this, it follows that dextranase would be an effective anticaries agent. Keyes (1968a) has written: "These findings strengthen the hypothesis that dextrans contribute to plaque formation and adhesiveness and indicate that appropriate enzymatic products can be of benefit in the treatment of plaque-associated dental infections."
Chapter 2

RESEARCH AIM

If the presence of dextran is essential for the development of tooth decay, then dextranase treatment of humans could be an important therapeutic measure. Some investigators (Fitzgerald, Keyes, Stout, and Spinell, 1968) have suggested "investigating the effects of dextranase on dental plaque and dental caries in humans." However, it would seem that the validity of this approach requires more rigorous examination than the preliminary experimental evidence afforded. The purpose of the work recorded in this thesis was to test the essentiality of dextran in the dental caries process. There are several ways to test this proposition:

1. The argument that dextran is a key requisite for tooth decay can be examined by a feeding experiment. If a microbe-bearing animal system is caries free on a given diet, then addition of dextran to this diet could help in evaluating the essentiality of dextran in the caries process. This was done by feeding Sprague-Dawley rats either ground Purina rat chow or ground Purina rat chow containing 10% dextran.

2. The dextran hypothesis can be tested by identification of a cariogenic organism which has no dextran synthesizing property. This was done experimentally by examining cariogenic Lactobacillus casei 4646 (Rosen, Lenney, and O'Malley, 1968) for the presence or absence of an extracellular dextranucrase or of extracellular polysaccharide of the dextran class.
3. Another way of evaluating the premise that dextran is essential in the caries process would be to develop and isolate a mutant of a dextran-synthesizing, cariogenic microorganism which no longer synthesized dextran. Introduction of the mutant organism into a sucrose-fed, gnotobiotic animal system should permit appraisal of the dextran hypothesis.

4. An alternative experiment would be to monoinfect gnotobiotic animals with a cariogenic bacterial strain. By feeding the infected gnotobiotes a diet containing carbohydrate other than sucrose, the induction or failure of induction of dental caries should aid in evaluating dextran essentiality.

5. Finally, isolation of a bacterial strain which synthesizes dextran from sucrose and colonizes the teeth of sucrose-fed animals but fails to induce carious lesions would help in the assessment of the dextran hypothesis.

Experimentally, approaches 1 and 2 above were performed and serve as the basis for this thesis. Approach 3 was attempted unsuccessfully. Other workers have attacked the problem from approaches 4 and 5. This information is included in the discussion section of this thesis.
Chapter 3

EFFECT OF DEXTRAN FEEDING ON CARIES EXPERIENCE IN RATS

Gibbons et al., (1966) have written: "Once a capsule and plaque forming micro-organism is present in the oral cavity, then other acidogenic bacteria present in the plaque could contribute to the decay process. This would explain the presence of highly acidogenic streptococci in large numbers in carious lesions, such as strain GS15, which in pure culture are unable to induce dental caries." This concept implied that dextran, present in the oral cavity and capable of forming insoluble precipitates with saliva and hydroxyapatite (Gibbons and Banghart, 1967) might allow oral, acidogenic organisms to colonize teeth and induce carious lesions. This reasoning suggested an experiment to test the role of dextran in dental caries. One group of Sprague-Dawley rats was fed ground Purina Laboratory Chow and another group of Sprague-Dawley rats was fed ground Purina Laboratory Chow containing 10% high molecular weight dextran. The Sprague-Dawley rat was chosen because:


MATERIALS AND METHODS

Forty-five, weanling (21 days), conventional, microbe-bearing Sprague-Dawley male rats were purchased from Zivic-Miller Laboratories. Animals were maintained in wire cages in temperature and humidity controlled quarters. After several days on pelleted Purina rat chow, the animals were allocated randomly to three groups of 15 animals each and fed the diets shown in table 1.

At the end of 73 days the 45 rats were sacrificed by decapitation. The heads were autoclaved, defleshed, and the teeth examined under a microscope for dental caries.

RESULTS

The growth curves and total dietary intake per animal for the three groups are shown in Figure 1. Group one demonstrated the largest average body weight associated with the smallest food intake per animal. The largest average food consumption (approximately 10% greater than group one) was required by the group two rats. This suggested that group two rats were unable to utilize the 10% Cellu flour as a nutrient. However, group three rats experienced nearly the same growth as group one and two rats with only a slight increase in food intake above that of group one. This observation implied that dextran was used as a nutrient by the group three rats. The presence of a rodent alpha 1,6' glucosidase or a microbiological dextranase in the rat gastro-intestinal system would have made additional glucose available from the dextran.

The development of significant caries in group three with no
Table 1

Dietary Groups for Dextran-Feeding Experiment

<table>
<thead>
<tr>
<th>Group</th>
<th>Ground, Purina rat chow</th>
<th>Addition</th>
<th>Tap water</th>
<th>Caries experience</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ad libitum</td>
<td>Nothing</td>
<td>Ad libitum</td>
<td>No detectable lesions</td>
</tr>
<tr>
<td>2</td>
<td>Ad libitum</td>
<td>10% Cellu flour</td>
<td>Ad libitum</td>
<td>No detectable lesions</td>
</tr>
<tr>
<td>3</td>
<td>Ad libitum</td>
<td>10% Dextran</td>
<td>Ad libitum</td>
<td>No detectable lesions</td>
</tr>
</tbody>
</table>

aEach group was composed of 15 male, Sprague-Dawley rats.

bPurina rat chow was ground in Quaker City F No. 4 corn mill.

cTap water contained 1 part per million fluoride.

dTeeth of animals were examined under binocular, variable 7 to 30 power Bausch and Lomb microscope with the aid of a light and a sharp dental explorer after animals were sacrificed and jaws defleshed.

eChicago Dietetic Supply House Inc.'s Cellu flour, finely ground cellulose, was mixed with the ground, Purina rat chow.

fSigma's 5-4OF (5 to 40 x 10^6 molecular weight) powdered dextran was mixed with the ground, Purina rat chow.
Figure 1. Rat Weight as a Function of Time. Each experimental point represents the total body weight for a group divided by 15. The weights at the right end of the curve were obtained by summing up the food intake for each group over the 73 days and dividing by 15. See table 1 for definition of the dietary groups.
cavities in groups one and two would have been meaningful. However, none of the 45 rats experienced any detectable tooth decay. There was no detectable plaque on the molars of any rat.
Rosen, Lenney, and O'Malley (1968) have reported that Lactobacillus casei, American Type Culture Collection number 4646, induced pit and fissure cavities in the molars of sucrose-fed gnotobiotic rats but "there was no accumulation of dental plaque on any of the teeth." This observation suggested that this organism did not synthesize an adhesive, extracellular polysaccharide yet was able to induce carious lesions. Lactobacillus casei 4646 was examined for an extracellular dextranase. The extracellular, sucrose-utilizing enzymes, including the dextranase, from Streptococcus FA1 served as controls. Streptococcus FA1 has been shown to induce pit and fissure cavities exclusively and cause moderate amounts of dental plaque in sucrose-fed, gnotobiotic rats (Fitzgerald, Jordan, and Stanley, 1960 and Fitzgerald and Jordan, 1968). Wood and Critchley (1966) showed Streptococcus FA1 synthesized extracellular dextran when grown on sucrose-containing medium and Wood (1967b) demonstrated an extracellular dextranase enzyme associated with glucose-grown Streptococcus FA1.

MATERIALS AND METHODS

Streptococcus FA1 was furnished by Dr. Robert Fitzgerald. Lactobacillus casei 4646 was purchased from the American Type Culture Collection. After initial receipt of the two pure strains of microorganisms, each strain was streak-plated on 0.04% NaHCO₃ supplemented
N. I. H. thioglycollate (Difco) 1.5% agar plates. Plates were examined for contamination. Representative colonies were selected from the plates and inoculated into N. I. H. thioglycollate (0.04% NaHCO₃ supplemented) broth tubes (16 x 125 mm screw cap). After overnight growth at 37°C, single loop (approximately 0.05 ml) inocula were taken from the broth tubes and introduced into N. I. H. thioglycollate (0.04% NaHCO₃ supplemented) 0.3% agar deeps (16 x 125 mm screw cap tubes) containing a pinch of CaCO₃. When growth was visible in the calcium carbonate, 0.3% agar deeps, they were removed from the 37°C incubator and stored in the refrigerator. The calcium carbonate, 0.3% agar deeps served as stock cultures. Every 10 to 14 days the two strains were grown in broth medium and then transferred to fresh calcium carbonate, 0.3% agar deeps. At approximately six month intervals the streptococcal strain was plated out on N. I. H. thioglycollate (0.04% NaHCO₃ supplemented) 1.5% agar plates and Mitis Salivarius (Difco) 1.5% agar plates. Similarly, the lactobacillus strain was plated on N. I. H. thioglycollate (0.04% NaHCO₃ supplemented) 1.5% agar plates and Lactobacillus Selective Agar (Baltimore Biological Company) 1.5% agar plates. The resulting colonies were examined for gross changes and contamination. Representative colonies were selected for the production of new stock cultures.

The preparative method for the extracellular dextransucrase was a modification of the procedures of Hehre (1955) and Wood (1967b). The stepwise plan for preparation of the extracellular enzymes is outlined below:
Cultures of Streptococcus FA1 or Lactobacillus casei 4646 were grown on N. I. H. thioglycollate broth (0.04% NaHCO₃ supplemented) to mid log phase as determined by turbidimetry at 610 nm.

Step 1 Bacterial cells were removed by centrifugation at approximately 11,000 x g for 30 minutes at 0-4°C.

Cold, supernatant fluid

Step 2 Supernatant fluid was passed immediately through a sterile 0.22 micron GS (general sterilization) Millipore filter into a sterile receiver.

Cold, supernatant filtrate was checked for presence of cells by plating duplicate 0.1 ml samples on N. I. H. thioglycollate (0.04% NaHCO₃ supplemented) agar and Mitis Salivarius (Difco) agar (for the streptococcal strain only) or Lactobacillus Selective Agar (for the lactobacillus strain only).

Step 3 Solid ammonium sulfate (0.37 g enzyme grade ammonium sulfate/ml supernatant fluid) was added with mechanical stirring while maintaining the lowered temperature with an ice bath.

Cold, half-saturated ammonium sulfate suspension was refrigerated overnight.

Step 4 Precipitate was collected by centrifugation at approximately 25,000 x g for 15 minutes at 0-4°C.

Precipitate

Step 5 Precipitate was washed with cold, half-saturated ammonium sulfate in 0.1% acetic acid (5 ml/50 ml Nalgene centrifuge tube). Precipitate was collected by centrifugation at approximately 25,000 x g for 15 minutes at 0-4°C.

Washed precipitate

Step 6 Washed precipitate was extracted with 0.025 M citrate pH 6.3 buffer (5 ml/50 ml Nalgene centrifuge tube) for at least 1 hour in the refrigerator. The resulting citrate solution was cleared by centrifugation at 25,000 x g for 15 minutes at 0-4°C.

Supernatant, citrate extract

Step 7 The supernatant, citrate extract (approximately 10 ml) was placed in dialysis material and dialyzed for approximately 48 hours against three 250 ml changes of 0.025 M citrate pH 6.3 buffer in the refrigerator with mechanical agitation.

Dialyzed, supernatant, citrate extract
Step 8 Dialyzed, supernatant, citrate extract was passed through a sterile 0.22 micron GS Millipore filter into a sterile receiver.

Filtered dialyzate was checked for the presence of cells by plating duplicate 0.1 ml samples on N. I. H. thioglycollate (0.04% NaHCO₃ supplemented) agar and Mitis Salivarius agar or Lactobacillus Selective Agar and then stored in sterile, screw-cap glass tubes in the refrigerator. The resulting preparations were designated:

FA1dial 1-23 and 46461-6glu

The stepwise plan for a modified preparative procedure for the extracellular enzymes from Lactobacillus casei 4646 grown on N. I. H. thioglycollate (0.04% NaHCO₃ supplemented) medium in which 5% sucrose had been substituted for glucose is outlined below:

Lactobacillus casei 4646 was grown on N. I. H. thioglycollate medium that contained 5% sucrose instead of glucose to mid log phase as determined by turbidimetry at 610 nm.

Step 1 Bacterial cells were removed by centrifugation at approximately 11,000 x g for 30 minutes at 0-4°C.

Cold, supernatant fluid

Step 2 Supernatant fluid was passed immediately through sterile 0.22 micron GS Millipore filter into sterile receiver.

Cold, supernatant filtrate was checked for presence of cells by plating duplicate 0.1 ml samples on N. I. H. thioglycollate (0.04% NaHCO₃ supplemented) and Lactobacillus Selective (LBS) Agar.

Step 3 Cold, supernatant filtrate (approximately 40 ml) was dialyzed against five 500 ml changes of 0.025 M citrate pH 6.3 buffer for 72 hours with agitation in the refrigerator.

Dialyzate

Step 4 Dialyzate was passed through sterile 0.22 micron GS Millipore filter into sterile receiver.

Filtered dialyzate was checked for presence of cells by plating duplicate 0.1 ml samples on N. I. H. thioglycollate and LBS agar. The preparation was stored refrigerated in a sterile, screw-cap glass tube labeled 46462-suc.
This modified approach anticipated the objections that:

1. *Lactobacillus casei* 4646 might synthesize extracellular, sucrose-utilizing enzymes and especially a dextranase only in the presence of substrate (i.e., the enzyme might be inducible) or

2. the sucrose-utilizing enzymes might not be precipitated by half-saturation with ammonium sulfate.

One of the assays used to determine dextranase activity was a modification of the one suggested by Hehre (1955). Dextranase activity was determined by measuring fructose (actually reducing sugar) liberated from sucrose.

\[ \text{SUCROSE} \xrightarrow{\text{DEXTRANASE}} \text{DEXTRAN} + \text{FRUCTOSE} \]

Table 2 page 29 shows the contents of reaction tubes which were run in duplicate and prepared from reagents tempered to 30°C. Each reaction tube was sampled (0.2 ml sample from each tube) immediately after adding and mixing the sucrose with the enzyme preparation. These samples were designated zero time samples. Each 0.2 ml sample was placed in a solution of 8.8 ml of distilled, deionized water and 1.0 ml of 0.04 N sodium hydroxide to terminate the enzyme reaction. At one and two hour intervals after the zero time sample, additional 0.2 ml samples were removed from the reaction tubes and the enzyme inactivated as before. Two milliliter samples from each of the resulting sodium hydroxide solutions were pipetted into 25 ml sugar tubes. A 1.5 ml aliquot of an alkaline copper solution (Somogyi, 1952) was added with mixing to each sugar tube containing the 2 ml sample from the sodium hydroxide inactivated reaction mixture. The solutions in the sugar tubes were heated in a boiling water bath for 15 minutes. The contents
of the sugar tubes were cooled by immersing the tubes in cold, tap water. One milliliter of an arsenomolybdate solution (Nelson, 1944) was added to each cooled sugar tube. The volume of the sugar tube solutions was adjusted to 25 ml by adding distilled, deionized water. The sugar tubes were closed with rubber stoppers. The solutions were then mixed by inverting the tubes several times. The color of the resulting solutions was developed for 1.5 hours at room temperature. Absorbances of the solutions were read at 750 nm using a Bausch and Lomb Spectronic 20 colorimeter (Marais, DeWit, and Quicke, 1966). The concentrations of reducing sugar were obtained from a standard curve which related absorbance to known amounts of fructose.

Another technique was developed to estimate dextranase activity. This spectrophotometric, coupled-enzyme system is a kinetic assay (as opposed to a fixed time assay) which can distinguish between glucose and fructose. Calbiochem's Glucose Stat-Pack reagent served as the basic material for the coupled-enzyme system. The Glucose Stat-Pack reagent contains ATP, hexokinase, NADP, glucose-6-phosphate dehydrogenase, and buffer (tris-succinate, pH 7.5, containing magnesium and L-aspartate). This combination of biochemicals is used to detect glucose by measuring spectrophotometrically the change in absorbance at 340 nm.

\[
\text{GLUCOSE} + \text{ATP} \xrightarrow{\text{HEXOKINASE}} \text{GLUCOSE-6-PHOSPHATE} + \text{ADP}
\]

\[
\text{GLUCOSE-6-PHOSPHATE} \xrightarrow{\text{GLUCOSE-6-PHOSPHATE DEHYDROGENASE}} \text{6-PHOSPHOGLUCONATE}
\]

\[
\text{NADP}^+ \xrightarrow{\text{DEHYDROGENASE}} \text{NADPH} + \text{H}^+
\]

The addition of phosphohexoseisomerase (isomerase) to the Glucose Stat-Pack reagent permits fructose detection.
Glucose Stat-Pack reagent was activated by adding 15.5 ml of distilled, deionized water to vial A which contained excess ATP, hexokinase, glucose-6-phosphate dehydrogenase, and buffer. The vial was capped and swirled gently to dissolve the powdered material. The entire solution in vial A was transferred to vial B which contained powdered NADP. Vial B was capped and gently swirled. The stability of the Glucose Stat-Pack reagent has been found to be approximately 2 hours at 30° C, 6 hours at 25° C, and 2 days at 4° C (Calbiochem technical insert).

The phosphohexoseisomerase was Sigma's grade III yeast enzyme. The enzyme was supplied as an ammonium sulfate suspension which contained 20 milligrams of protein per milliliter of suspension. One milligram of protein converted 425 micromoles of fructose-6-phosphate to glucose-6-phosphate at pH 7.4 and 25° C. A 0.1 ml sample of the ammonium sulfate suspension was centrifuged at approximately 11,000 x g for 10 minutes at 0° C. The supernatant fluid was eliminated. The resulting precipitate was dissolved in one milliliter of distilled, deionized water. This isomerase solution was stored on ice until an aliquot was added to a reaction cuvette. The phosphohexoseisomerase was prepared fresh daily before use and a 0.1 ml aliquot of the water solution contained activity capable of converting up to 85 micromoles of fructose-6-phosphate per minute. The water solution of isomerase was stable for two days when stored on ice or refrigerated. The small amount of ammonium sulfate present in the water solution of the isomerase did not affect the detection of known amounts of glucose or fruc-
tose by the coupled-enzyme system. The ammonium sulfate also did not alter the release of reducing sugar from sucrose by the extracellular enzymes from *Streptococcus* FA1 as measured by the reducing sugar (modified Somogyi) method.

A number of points had to be investigated before the spectrophotometric, coupled-enzyme system was accepted as a valid assay for dextranulcrase activity. Operating within the conditions of the spectrophotometric, coupled-enzyme system, the following points were established experimentally:

1. Known amounts of glucose were detected quantitatively using a solution containing Glucose Stat-Pack reagent.

2. There was no change in absorbance in a solution containing Glucose Stat-Pack reagent (without phosphohexoseisomerase) when fructose was added.

3. Known amounts of fructose were detected quantitatively using a solution containing Glucose Stat-Pack reagent modified by the addition of phosphohexoseisomerase.

4. The rates of detection of glucose and fructose were additive under the conditions of the spectrophotometric, coupled-enzyme system. In other words, the rate of detection of a known amount of glucose plus the rate of detection of a known amount of fructose equalled the rate of detection using a solution containing both glucose and fructose in the same concentrations as in the individual solutions.

5. The presence of the extracellular, bacterial preparation from *Streptococcus* FA1 did not alter significantly the quantitative detection of glucose or fructose using solution containing Glucose Stat-Pack reagent or Glucose Stat-Pack reagent with phosphohexoseisomerase.
6. The bacterial enzymes were the rate limiting factors in the release of glucose and fructose from sucrose since doubling the concentration of Glucose Stat-Pack reagent and/or phosphohexoseisomerase in the reaction solution did not increase the rate of detection of either sugar.

7. Fructose release by the extracellular preparation from *Streptococcus* FA1, FA1\textsuperscript{dial 8-9}, was a linear function of protein concentration over the range of approximately 6 μg to 600 μg protein (N = 9 and r = 0.99).

8. Examination of the FA1\textsuperscript{dial 8-9} preparation at two different times one week apart showed no change in the specific activity of the fructose releasing ability.

Activated Glucose Stat-Pack reagent, water-dissolved phosphohexoseisomerase (where appropriate), appropriate bacterial enzyme preparation, and adequate distilled, deionized water to give a final volume of 3.0 ml were added to quartz cuvettes (Hellma QS internally matched, 10 mm light path). The resulting solution was gently mixed by covering and inverting. The cuvettes were placed in a holder in the cuvette compartment of the Gilford-2000 recording spectrophotometer. Zero absorbance was established with a cuvette containing distilled, deionized water. Since the dextran sucrase unit of activity (DSU) was defined at 30° C, the cuvette compartment was thermostated to operate at this temperature. It was found that the fluids being used in the cuvettes (bacterial enzyme preparations and isomerase were stored on ice until added to the cuvettes) equilibrated to 30° C within four to five minutes in the Gilford-2000 cuvette compartment. Therefore, the absorbance was monitored for approximately this time
period before sucrorse (at 30°C) was added to initiate the reaction. Glucose release from sucrose was assumed to be a measure of the extracellular levansucrase from *Streptococcus* FA1. Fructose release from sucrose was assumed to be a measure of the extracellular dextran sucrase from *Strep.* FA1.

To substantiate the dextran sucrase activity, dextran was isolated from a reaction mixture which was similar to the conditions used in the spectrophotometric, coupled-enzyme system. The following reaction mixture was prepared:

- 7.75 ml distilled, deionized water
- 6.5 ml Glucose Stat-Pack reagent (heated in a boiling water bath for 10 minutes)
- 0.25 ml FA1\_dial filter 1-23
- 0.5 ml 30% sucrose in distilled, deionized water

The reagents were mixed and filtered through a 0.22 micron GS Millipore filter into a sterile receiver. Duplicate 0.1 ml samples were removed and plated to gauge the cell free condition. The reaction was allowed to proceed at room temperature for two weeks. Ethanol (95%) was added to give a final ethanol concentration (v/v) of 45% to precipitate any dextran (Mehre and Neille, 1946, Jeannes, 1965, Wood and Critchley, 1966, and Dahlquist et al., 1967). After one hour in the refrigerator, the precipitate was collected by centrifugation (18,000 x g for 30 minutes at 0°C). The precipitate was dissolved in 5 ml of 1 N hydrochloric acid. The acid solution was placed in a closed glass tube and heated in a boiling water bath for two hours as were appropriate glucose and fructose standards in 1 N hydrochloric acid. Samples of the acid hydrolysate of the 45% ethanol precipitate were
applied to Whatman #1 filter papers and allowed to dry overnight. Two solvent systems (n-butanol:pyridine:water; 2:5:7 and ethyl acetate: acetic acid: water; 9:2:2) according to the procedure of Dahlquist et al., (1967) were used to develop the filter papers in an ascending direction. Ammoniacal silver nitrate (Litwack, 1960) was used as the locating reagent after the developed chromatograms had been dried.

In another experiment to verify dextran synthesis, the following reaction mixture was prepared:

11 ml distilled, deionized water
13 ml of Glucose Stat-Pack reagent (inactivated by storing at room temperature overnight)
1 ml distilled, deionized water solution of phosphohexoseisomerase
1 ml PA1 dial 8-9
4 ml 30% sucrose in distilled, deionized water

The reagents were mixed and filtered through a 0.22 micron GS Millipore filter into a sterile receiver. Duplicate 0.1 ml samples were removed and plated to measure the cell free condition. After two days incubation at room temperature duplicate 0.1 ml samples were again plated out to verify the cell free condition. Two and one-half volumes of 95% ethanol were added and the suspension stored overnight in the refrigerator. The precipitate was collected by centrifugation at 5,000 x g for 15 minutes at 0-4°C. The precipitate was dissolved in distilled, deionized water and reprecipitated with 2.5 volumes of 95% ethanol. The precipitate was collected as before and again dissolved in 5 ml of distilled, deionized water. This solution was dialyzed against 200 ml of distilled, deionized water with agitation overnight in the refrigerator. Aliquots of the dialyzed material were incubated
at 30° C with a distilled, deionized water solution of dextranase (Worthington). The incubation mixture was sampled at zero time immediately after mixing the reagents. The zero time sample contained no glucose as determined by the Glucose Stat-Pack reagent. The incubation mixture was sampled approximately one and two hours later. These samples were tested for glucose using the Glucose Stat-Pack reagent.

Protein in the extracellular preparations was estimated by a biuret method (Layne, 1957). Absorbances of the biuret-protein solutions were read at 550 nm using a Bausch and Lomb Spectronic 20 colorimeter. Protein concentrations were obtained from a standard curve which related absorbance to known amounts of Sigma's crystalline, lyophilized bovine serum albumin.

RESULTS

Table 2 presents the results of the survey of the extracellular preparations for the ability to release reducing sugar from sucrose. Extracellular material from glucose or sucrose grown Lactobacillus casei 4646 contained no enzymes, including a dextranose, which released reducing sugar from sucrose within the growth, isolation, and assay conditions of the experiment. The cariogenic L. casei 4646 resembled the non-cariogenic Streptococcus JR81G (Jordan, 1965) in that neither organism produced extracellular enzymes which released reducing sugar from sucrose under the conditions used. Sucrose-grown, non-cariogenic Strep. JR81G also was found not to possess any extracellular sucrose-utilizing enzymes which released reducing sugar although this information is not presented in table 2. This experiment showed that Streptococcus FA1 possesses an extracellular enzyme
### Table 2

Reducing Sugar Release from Sucrose by Extracellular Preparations

<table>
<thead>
<tr>
<th>Organism</th>
<th>Reaction tube contents at 30° C in ml</th>
<th>Amoles reducing sugar released in one hour per 3 ml of incubation mixture</th>
<th>Amoles reducing sugar released in two hours per 3 ml of incubation mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extracellular preparation 0% sucrose 0.025 M citrate pH 6.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>0.5</td>
<td>3.75 (0.024)</td>
<td>6.46 (0.021)</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>1.0</td>
<td>5.00 (0.016)</td>
<td>7.91 (0.013)</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>2.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>1.0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

( ) = specific activity expressed in umoles of sucrose utilized per minute per milligram of protein

a Preparation contained 5.2 mg protein per ml

b Preparation contained 4.1 mg protein per ml

c Preparation contained 2.8 mg protein per ml

d Preparation contained 3.8 mg protein per ml
(or enzymes) which released reducing sugar from sucrose. The release of reducing sugar from sucrose by the extracellular preparation from Strep. FA1 increased with time and enzyme concentration although not linearly at the times and concentrations employed.

Figure 2 shows the results of the spectrophotometric, coupled-enzyme assay for the release of glucose and/or fructose from sucrose by the extracellular preparation, FA1\textsuperscript{dial} \textsubscript{filt} 1-23 from glucose-grown Streptococcus FA1. Curve one in Figure 2 corresponds to the absorbance of distilled, deionized water. Curve two represents the absorbance of a solution which contained no isomerase and therefore detected only the release of glucose from sucrose by the extracellular preparation from Strep. FA1. Not too surprisingly, Strep. FA1 possesses an extracellular enzyme which liberated glucose from sucrose. This enzyme was assumed to be a levensucrase since Wood and Critchley (1966) had shown that Strep. FA1 synthesized an extracellular levan when grown on sucrose medium. Curve three depicts the absorbance of a solution which contained isomerase and therefore detected glucose plus fructose release from sucrose. The difference between curves three and two represents the release of fructose from sucrose by the extracellular preparation from Strep. FA1. The release of fructose was assumed to be a measure of the dextransucrase. The unequal rates of release of glucose and fructose from sucrose by the extracellular preparation argues against the explanation that an invertase (or sucrase) was the only active enzyme present in the extracellular preparation. Curve four in Figure 2 presents the absorbance of a solution which contained isomerase and double the concentration of the extracellular preparation from Streptococcus FA1, FA1\textsuperscript{dial} \textsubscript{filt} 1-23, compared to the solutions
Figure 2. Spectrophotometric, Coupled-Enzyme Assay for Glucose and Fructose Release from Sucrose by an Extracellular Preparation from Glucose-Grown *Streptococcus* FA1. Absorbance at 340 nm was recorded using a Gilford-2000. Cuvette contents corresponding to the curves are described in table 3. Reaction was initiated by adding sucrose.
Table 3

Spectrophotometric, Coupled-Enzyme Assay for Glucose and Fructose Release from Sucrose by an Extracellular Preparation from Glucose-Grown Streptococcus FA1 (Cuvette Contents for Figure 2)

<table>
<thead>
<tr>
<th>Curve</th>
<th>Cuvette contents at 30° C in ml:</th>
<th>Distilled, deionized water</th>
<th>Glucose Stat-Pack</th>
<th>Isomerase</th>
<th>FA\textsubscript{dial}\textsubscript{filt} 1-23</th>
<th>30% sucrose</th>
<th>Specific activity\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.575</td>
<td>1.3</td>
<td></td>
<td>0.025</td>
<td>0.1</td>
<td>0.0100</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.475</td>
<td>1.3</td>
<td>0.1</td>
<td>0.025</td>
<td>0.1</td>
<td>0.0154</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.45</td>
<td>1.3</td>
<td>0.1</td>
<td>0.05</td>
<td>0.1</td>
<td>0.0139</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}FA\textsubscript{dial}\textsubscript{filt} 1-23 contained 5.2 mg protein per ml preparation

\textsuperscript{b}Specific activity in \textmu moles of sucrose utilized per minute per milligram of protein
in the cuvettes corresponding to curves two and three. The specific activities calculated for total sucrose utilization from curves three and four (see table 3) are in fair agreement with those seen in table 2 page 29 although the reaction pH's and sucrose concentrations were different. Using the difference of curves two and three, a value of 0.0054 μmoles of sucrose utilized per minute per milligram of protein was calculated for the specific activity for the extracellular, fructose-releasing activity from _Streptococcus_ FA1 (assuming no invertase activity).

An extracellular preparation from sucrose-grown _Streptococcus_ FA1 was also found to release both glucose and fructose from sucrose by the coupled-enzyme system although this information is not presented.

Two other experiments should be noted here although the data are not presented. The extracellular preparation from glucose-grown _Streptococcus_ FA1 did not release glucose when incubated with a 0.01% solution of Sigma's 5-40F dextran or a 10% solution of lactose. These results suggested that the preparation did not contain a dextranase enzyme and that the enzymes in the preparation were specific for the disaccharide, sucrose.

Paper chromatography of the acid-hydrolyzed, ethanol-precipitable material from the reaction mixture simulating the spectrophotometric, coupled-enzyme system revealed glucose as the only sugar. This showed that fructose release from sucrose by the extracellular preparation from _Streptococcus_ FA1 was related to polyglucose synthesis. It was found also that glucose, measured with the Glucose Stat-Pack reagent, was released by dextranase from the ethanol-precipitable material isolated from the reaction mixture which simulated the spectrophoto-
metric, coupled-enzyme system. These results suggested that the fructose release from sucrose by the extracellular preparations from \textit{Streptococcus FA1} corresponds to dextran synthesis.

Figure 3 presents the results of the spectrophotometric, coupled-enzyme assay for the release of glucose and/or fructose from sucrose by the extracellular preparation, $4646^{2-8}_{\text{suc}}$ from sucrose-grown \textit{Lactobacillus casei} 4646. This preparation contained the total extracellular, non-dialyzable material as outlined on page 20. Curve one corresponds to the absorbance of a distilled, deionized water blank. Curve two represents the absorbance of a reagent blank which contained no enzyme preparation from \textit{L. casei} 4646. Curve two shows that the 30% sucrose solution although incubated at 30°C was not hydrolyzed significantly when prepared daily. Curve three represents the absorbance of a solution which was capable of detecting glucose and fructose release from sucrose by the preparation (0.7 mg $4646^{2-8}_{\text{suc}}$ protein) from sucrose-grown \textit{L. casei} 4646. Curve four corresponds to the absorbance of a solution which contained a doubled amount of extracellular preparation (1.4 mg protein) from sucrose-grown \textit{L. casei} 4646. The absorbances of the solutions corresponding to curves three and four demonstrate no increase above the absorbance exhibited by the solution corresponding to curve two, the reagent blank. There is no increase in absorbance with increasing concentration of extracellular preparation, $4646^{2-8}_{\text{suc}}$. This suggested that \textit{L. casei} 4646 possesses no extracellular enzymes which release glucose or fructose from sucrose.

Addition of extracellular preparation (0.52 mg $\text{FA1}^{\text{dial}}_{\text{fillt}}$ 1-23 protein) from glucose-grown \textit{Streptococcus FA1} at approximately 23 minutes
Figure 3. Spectrophotometric, Coupled-Enzyme Assay for Glucose and Fructose Release from Sucrose by an Extracellular Preparation from Sucrose-Grown Lactobacillus casei 4646. Absorbance at 340 nm was recorded using a Gilford-2000. Cuvette contents corresponding to the curves are described in table 4. Reaction was initiated by adding sucrose. FA1dial 1-23 was added at 23 minutes to demonstrate functional detection systems.
Table 4

Spectrophotometric, Coupled-Enzyme Assay for Glucose and Fructose Release from Sucrose by an Extracellular Preparation from Sucrose-Grown Lactobacillus casei 4646
(Cuvette Contents for Figure 3)

<table>
<thead>
<tr>
<th>Curve</th>
<th>Distilled, deionized water</th>
<th>Cuvette contents at 30°C in ml:</th>
<th>30% sucrose</th>
<th>Specific activity [b]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>Glucose Stat-Pack Isomerase 4646(_{2-8}) suc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>3.0</td>
<td>1.3</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>1.25</td>
<td>1.3</td>
<td>0.1</td>
<td>0.25</td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>1.3</td>
<td>0.1</td>
<td>0.0</td>
</tr>
</tbody>
</table>

\[a\] 4646\(_{2-8}\) suc contained 2.8 mg protein per ml preparation

\[b\] Specific activity in umoles of sucrose utilized per minute per milligram of protein

FA\(_{1}\)dial 1-23 added to show functional detection systems contained 5.2 mg protein per ml preparation. A 0.1 ml aliquot (0.52 mg protein) of the FA1 preparation was added to each of the cuvettes corresponding to curves 2, 3, and 4 at the 23 minute mark in Figure 3.
in Figure 3 illustrated that all detecting systems were functional.

The extracellular preparation from glucose-grown *Lactobacillus casei* 4646 (4646^{1-6}_{glu}) also failed to release glucose or fructose from sucrose, although the detailed data from the spectrophotometric, coupled-enzyme assay are not presented.
EXAMINATION OF LACTOBACILLUS CASEI 4646 FOR EXTRACELLULAR DEXTRAN

The results presented in chapter 4 suggested that Lactobacillus casei 4646 probably does not synthesize dextran from sucrose. Hammond's (1969) observation that glucose-grown Lactobacillus casei 32-1+ yielded a cell-free preparation which had dextran sulphur activity illustrated that some L. casei possess dextran synthesizing capabilities. Although the approach used in chapter 3 would have detected an extracellular, L. casei 4646 dextran sulphurase, it was believed, in light of Hammond's (1969) work, that a more rigorous examination of L. casei 4646 was needed. There was the possibility that L. casei 4646 might have a cell-associated dextran sulphorase which was bound tightly enough to prevent loss into the external environment yet could synthesize extracellular dextran from sucrose. There was also the possibility that L. casei 4646 might possess an intracellular dextran sulphorase and excrete dextran into the external environment. Therefore, sucrose-grown L. casei 4646 was examined for the presence of dextran. Sucrose-grown Streptococcus FA1, a known dextran synthesizer, was examined simultaneously to provide a control.

MATERIALS AND METHODS

The method of dextran isolation was that described by Wood and Critchley (1966). This procedure would isolate dextran or levan from any of the organisms listed in table 6 chapter 8. A one hundred milli-
A liter portion of Brain-Heart Infusion broth (Difco) supplemented with 10% sucrose was placed inside dialysis material (Fisher #8-667-2). The dialysis bag containing the above medium was positioned in a screw-cap flask containing 400 ml of the same sterile medium as the external environment. The sterile medium inside the dialysis bag was inoculated with 10 ml of an overnight culture of the appropriate bacteria in Brain-Heart Infusion broth. The inoculated system was incubated at 37°C for four days. The material in the dialysis bag was poured into a beaker and 2.5 volumes of cold, 95% ethanol were added. The resulting precipitate was collected by centrifugation (2500 x g) for 15 minutes at 0-4°C. The supernatant fluid was decanted and the precipitate suspended in 50 ml of distilled, deionized water. This procedure was repeated two additional times. The precipitate, suspended in water, was centrifuged at 2500 x g for 15 minutes at 0-4°C. This operation removed essentially all the cells in the preparations from both strains. The supernatant, cell-depleted fluid was then deproteinized by adding chloroform and tertiary amyl alcohol with shaking according to the method for Sevag (1954). After separation, 2.5 volumes of cold, 95% ethanol were added to the aqueous layer from the deproteinization system. At this point the fluid from the Streptococcus FA1 culture gave an abundant, adhesive, white precipitate. The fluid from the Lactobacillus casei 4646 culture yielded no precipitate employing identical conditions. Increasing the ethanol concentration and refrigerating this solution for several days failed to produce a precipitate in the preparation from the sucrose-grown L. casei 4646.

The ethanol-induced precipitate from the Streptococcus FA1


preparation after deproteinization was collected by centrifuging at 2500 x g for 15 minutes at 0-4° C. This precipitate was taken up in 50 ml of distilled, deionized water. Two and one-half volumes of cold, 95% ethanol were added to the syrupy, water solution and the resulting precipitate was collected by centrifugation at 2500 x g at 0-4° C. This precipitate was again dissolved in 50 ml of distilled, deionized water. Ninety-five percent ethanol was added with stirring to bring the ethanol concentration to 45% (v/v) in the water solution. The resulting suspension was left overnight in the refrigerator. After decanting the supernatant fluid, the sticky precipitate was washed three times with approximately 50 ml of 95% ethanol. After washing, the precipitate was dissolved in 25 ml of distilled, deionized water. This solution was frozen and lyophilized. The resulting white powder was stored at room temperature in a dessicator.

Approximately 1% solutions of the lyophilized, white, 45% ethanol precipitable material from sucrose-grown Streptococcus FA1, glucose (Baker's reagent grade monohydrate), and fructose (Pfanstiehl's anhydrous) were prepared in 3 N hydrochloric acid. One milliliter aliquots of each solution were heated in screw-cap, glass tubes at approximately 100° C for 3 hours. The resulting solutions were evaporated on watch glasses over a steam bath. The dried material on the watch glasses was taken up in 1 ml of distilled, deionized water. Small aliquots of the resulting solutions were applied to Whatman #1 filter paper. Two solvent systems (phenol:water; 4:1 and ethyl acetate: pyridine:water; 125:50:40) were used to develop the ascending paper chromatograms. After development and drying, the paper chromatograms were sprayed with ammoniacal silver nitrate (Litwack, 1960) locating
Additional proof of the nature of the lyophilized, 45% ethanol precipitable material isolated from sucrose-grown Streptococcus FA1 was obtained by incubating at 30°C aliquots of a 0.5% distilled, deionized water solution of the material with a dextranase (Worthington) preparation. The incubation mixture was sampled at zero time immediately after mixing the reagents. The zero time sample contained no glucose as determined with the Glucose Stat-Pack reagent. The incubation mixture was sampled approximately one and two hours later. These samples were tested for glucose using the Glucose Stat-Pack reagent.

Potassium bromide disks containing lyophilized, 45% ethanol precipitable material isolated from sucrose-grown Streptococcus FA1 and Sigma's 5-40F dextran were examined for infrared absorption using a Perkin-Elmer model 257 infrared spectrophotometer. A KBr disk containing a sample of lyophilized, 45% ethanol precipitable material isolated from a cell-free reaction mixture similar to the spectrophotometric, coupled-enzyme system was also analyzed for infrared absorption.

A 0.5% distilled, deionized water solution of the lyophilized, 45% ethanol precipitable material from sucrose-grown Streptococcus FA1 was examined in a Unicam SP. 800 double beam, scanning spectrophotometer over the wavelength range of 250 nm to 700 nm.

An approximately 1% distilled, deionized water solution of the lyophilized, 45% ethanol precipitable material from sucrose-grown Streptococcus FA1 was examined for protein using the biuret method (Layne, 1957) described on page 28.
The lyophilized, 45% ethanol precipitable material from sucrose-grown *Streptococcus* FA1 was analyzed for carbon, hydrogen, oxygen, and nitrogen after drying at 80°C by a commercial laboratory, Micro-Tech Laboratories in Skokie, Illinois.

Distilled, deionized water solutions of the lyophilized, 45% ethanol precipitable material from sucrose-grown *Streptococcus* FA1 were subjected to sedimentation analysis in a Spinco model E ultracentrifuge at approximately 52,640 rpm employing the analytical D rotor and the Schlieren optics.

**RESULTS**

Sucrose-grown *Lactobacillus casei* 4646 synthesized no extracellular dextran within the growth and isolation conditions employed. (*L. casei* 4646, grown in lactose or maltose-containing environments, also failed to produce extracellular, ethanol precipitable material.)

Glucose was the only monosaccharide present after paper chromatography of the acid hydrolysate of the lyophilized, 45% ethanol precipitable material from *Streptococcus* FA1.

Dextranase (Worthington) released glucose linearly over approximately 2 hours as measured by the Glucose Stat-Pack reagent from a solution of the lyophilized, 45% ethanol precipitable material from *Streptococcus* FA1.

Curve FA1 in Figure 4 presents the infrared transmittance from a KBr disk containing lyophilized, 45% ethanol precipitable polyglucose isolated from sucrose-grown *Streptococcus* FA1. Curve FA1 depicts the infrared absorption of the KBr disk containing lyophilized, 45% ethanol precipitable material produced from sucrose by the cell-free,
Figure 4. Infrared Spectrograms. Curves 1a and 1b are the transmittances of KBr disks containing two different concentrations of Sigma's 5-40 x 10^6 molecular weight dextran. Curve FA1cf is the transmittance of a KBr disk containing dextran synthesized by the cell-free, extracellular enzyme from Strep. FA1. Curve FA1 is the transmittance of a KBr disk containing dextran isolated from sucrose-grown Strep. FA1.
extracellular enzyme preparation, FA\textsubscript{dial} \textsuperscript{8-9}, under conditions simulating the spectrophotometric, coupled, enzyme assay described in chapter 3. Curves 1a and 1b represent the infrared patterns from KBr disks containing two different concentrations of a commercial dextran, Sigma's 5-40 x 10\textsuperscript{6} molecular weight dextran from a \textit{Leuconostoc mesenteroides}. Curves FA\textsubscript{1} and FA\textsubscript{1 cf} appear to be identical to each other and are similar to curves 1a and 1b. However, curves FA\textsubscript{1} and FA\textsubscript{1 cf} conform more closely with the infrared spectrum given for the dextran isolated from cariogenic \textit{Streptococcus} OM2-176 (Guggenheim and Schroeder, 1967).

The distilled, deionized water solution of the dextran isolated from sucrose-grown \textit{Streptococcus} FA\textsubscript{1} exhibited no absorption peaks over the wavelength range of 250 nm to 700 nm. There was also no biuret reactive material detectable in the distilled, deionized water solution of the dextran isolated from sucrose-grown \textit{Strep.} FA\textsubscript{1}.

Carbon, hydrogen, oxygen, and nitrogen composition of the dextran isolated from sucrose-grown \textit{Streptococcus} FA\textsubscript{1} is given in table 5. The nitrogen was probably dextran-dextranucrase bound to the dextran.

Dextran, isolated from sucrose-grown \textit{Streptococcus} FA\textsubscript{1}, exhibited the following behavior during ultracentrifugation:

1. A single peak was detected at each dextran concentration over the concentration range of 0.189\% to 0.889\% dextran. All peaks were symmetrical.

2. The peaks exhibited spreading with increasing time in the centrifugal field.

3. The peak corresponding to the lowest dextran concentration (0.189\%) moved as a single peak during 45 minutes of centrifugation. Figure 5 shows the sedimentation coefficients plotted as a function of
Table 5
Elemental Composition of Dextran from *Streptococcus* FA1

<table>
<thead>
<tr>
<th></th>
<th>% weight loss by drying at 80°C</th>
<th>% Carbon</th>
<th>% Hydrogen</th>
<th>% Oxygen</th>
<th>% Nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextran from <em>Streptococcus</em> FA1</td>
<td>5.36</td>
<td>43.73</td>
<td>6.30</td>
<td>49.31</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>5.51</td>
<td>43.68</td>
<td>6.26</td>
<td>49.10</td>
<td>0.05</td>
</tr>
<tr>
<td>Theoretical values (<em>C₆H₁₀O₅</em>)ₙ</td>
<td></td>
<td>44.45</td>
<td>6.22</td>
<td>49.34</td>
<td></td>
</tr>
<tr>
<td>Ranges of values given in the literature&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.8 to 41.1</td>
<td>6.4 to 6.7</td>
<td>45.1 to 46.8</td>
<td>0.62 to 1.39</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Dahlquist et al., 1967
Figure 5. Sedimentation Coefficients as a Function of Dextran Concentration. Dextran concentration was corrected for % weight loss by drying at 80°C as shown in table 5.
dextran concentration. The linear extrapolation gives a limiting value of 3.1 svedbergs for this dextran at zero concentration. Using 0.6 for the partial specific volume and $0.52 \times 10^{-7}$ for the diffusion coefficient of dextran (Grönwall and Ingleman, 1944), a molecular weight of approximately 360,000 was obtained for the dextran from sucrose-grown Strep. FA1. This molecular weight must be considered, at best, an estimate for the following reasons:

1. Low molecular weight material may have been lost during growth of Strep. FA1 within the dialysis bag.

2. The effect of isolation on the size of the dextran molecules has not been evaluated. For example, the isolation procedures may have resulted in the mechanical degradation of the very high molecular weight species.

3. The heterogeneity of the isolated dextran has not been evaluated. Tanford (1961) has written that "... polysaccharides quite generally show a large difference between $M_n$ and $M_w$, indicative of molecular weight heterogeneity." Tanford (1961) also has pointed out that "it is generally considered preferable to use osmotic pressure, light scattering, sedimentation equilibrium, or Archibald's method for heterogeneous mixtures . . . ."

Therefore, the only conclusion made from results of the ultracentrifugation study is that the dextran, isolated from sucrose-grown Strep. FA1, is a relatively high molecular weight polymer (i.e., several hundred glucose molecules).
Chapter 6

ADHERENT GROWTH PROPERTIES OF STREPTOCOCCUS FA1 AND LACTOBACILLUS CASEI 4646 ON SUCROSE-CONTAINING MEDIUM

Jordan and Keyes (1966) demonstrated that Streptococcus FA1 "formed heavy bacterial plaques on ... stainless-steel wires ..." when utilizing a medium containing 5% sucrose. Fitzgerald, Spinell, and Stoudt (1968) used dextranase preparations from Penicillium funiculosum NRRL 1768 to remove the adherent growth produced by sucrose-grown Strep. FA1 on stainless-steel wires. This experiment demonstrated that extracellular dextran was the factor responsible for the adherence of Strep. FA1 to the stainless-steel wire. The results described in chapter 4 and 5 of this thesis predicted that Strep. FA1 and L. casei 4646 should have different adherent growth properties when grown on sucrose medium. In other words, the ability or lack of the ability to synthesize an extracellular dextran from sucrose should be translated into a difference in the adherent growth properties of these two organisms. Since L. casei 4646 has no dextran synthesizing property, this organism should not exhibit an in vitro adherent growth characteristic on sucrose medium as does Strep. FA1. L. casei 4646 and Strep. FA1 were grown on sucrose-containing medium in tubes carrying stainless-steel wires to test this prediction.

MATERIALS AND METHODS

The technique used to test the prediction was a modification of the one given by Jordan and Keyes (1966). The test was initiated by
inoculating 10 ml of sterile culture medium, N. I. H. thioglycollate (0.04% NaHCO₃ supplemented) broth with the appropriate carbohydrate, with 0.1 ml of an overnight culture of the appropriate bacteria. The 16 x 125 mm glass tube containing the inoculated medium was closed with a sterile, rubber-stopper assembly from which a stainless-steel wire (20 gauge stainless-steel orthodontic wire) extended into the medium. Bacterial growth was allowed to proceed to the stationary phase. The rubber stopper carrying the wire was then transferred to a fresh tube of sterile medium. This procedure was repeated using the material on the wire as an inoculum until 10 tubes of medium had been exhausted.

RESULTS

Figure 6 shows the results of this experiment. Tube one in Figure 6 illustrates the adherent growth (simulating dental plaque) which formed on the stainless-steel wire when Streptococcus FA1 developed in a medium containing 5% sucrose as the only carbohydrate. This material became visible during growth in the third tube of medium and was not displaced by vigorous shaking of the tube.

Tube two shows Streptococcus FA1 grown on the same medium except glucose was substituted for sucrose. Strep. FA1 grew well on this medium as evidenced by the turbidity of the cells settling to the bottom of the tube but failed to produce an adherent layer of cells on the wire. This failure illustrated the essentiality of sucrose for the in vitro formation of a tenacious plaque.

Tube three substantiates the prediction that Lactobacillus casei 4646 would not exhibit an adherent growth characteristic analogous to
Figure 6. Adherent Growth Properties. Medium used was composed of the ingredients found in N. I. H. thioglycollate (0.04% NaHCO₃ supplemented) broth with the appropriate carbohydrate added.
that of Streptococcus FA1 in the presence of sucrose-containing medium. Tube four demonstrates that the non-cariogenic Streptococcus JR8LG does not exhibit an adherent growth property when grown on a sucrose-containing medium. This is consistent with the finding that Strep. JR8LG has no extracellular sucrose-utilizing enzymes which release glucose and/or fructose. The cariogenic L. casei 4646 resembled the non-cariogenic Strep. JR8LG in its adherent growth character on sucrose-containing medium. L. casei 4646 also failed to produce visible, adherent layers of cells on stainless-steel wires when grown on glucose, fructose, maltose, or lactose-containing media, although this organism grew well in these carbohydrate environments. These results are consistent with the finding that the cariogenic L. casei 4646 has no extracellular, dextran-synthesizing ability.
Chapter 7

ATTEMPTED MUTANT PRODUCTION

Perhaps the most rigorous test of the dextran hypothesis would be the development of a mutant of a dextran-synthesizing, cariogenic microorganism which no longer synthesized dextran. Observation of the caries experience of sucrose-fed, gnotobiotic rats infected with such a mutant should permit acceptance or rejection of the dextran hypothesis.

MATERIALS AND METHODS

A log phase culture of *Streptococcus* FA1 was removed from the N. I. H. thioglycollate (0.04% NaHCO$_3$ supplemented) broth medium by centrifugation at approximately 11,000 x g for 15 minutes at 0-4° C. This pellet was suspended in 10 ml of sterile radiation buffer (0.7% K$_2$HPO$_4$, 0.2% KH$_2$PO$_4$, and 0.004% MgSO$_4$·7H$_2$O in distilled, deionized water). The suspension was poured into a sterile petri dish containing a sterile, magnetic stirring bar. The petri dish was placed on a magnetic stirrer approximately 24 inches below a General Electric G15T8, 15 watt germicidal ultraviolet lamp in a hood. The petri-dish cover was removed and the bacterial suspension irradiated for 15 minutes while stirring. The irradiated bacterial suspension was sampled in a darkened room and 0.1 ml aliquots were spread-plated on Mitis Salivarius (Difco) agar. The plates were incubated at 37° C under an atmosphere of 95% N$_2$-5% CO$_2$. Wild-type *Strep.* FA1 has a
characteristic colony morphology on Mitis Salivarius agar under these conditions (Jordan, Englander, and Lim, 1969). After 2 days incubation, the plates were examined. Two colonies were found which had an altered colony morphology. Stock cultures from these two colonies were produced in N. I. H. thioglycollate (0.04% NaHCO$_3$ supplemented) 0.3% agar deeps as described in chapter 4 page 18. Extracellular enzyme preparations were obtained from these two strains as described for the enzyme preparation from Strep. FA1 in chapter 4 pages 19 and 20. These enzyme preparations were examined for their ability to release glucose and fructose from sucrose as measured with the spectrophotometric, coupled-enzyme assay described in chapter 4 pages 22 to 26.

RESULTS

Both organisms which exhibited altered colony morphology after irradiation had extracellular enzymes which released glucose and fructose from sucrose at unequal rates. This result suggested that a change in colony morphology on Mitis Salivarius agar was not necessarily related to changes in the synthesis of extracellular enzymes which release glucose and fructose from sucrose. This approach is still attractive if a technique can be developed to identify more readily a mutant which does not synthesize dextran.
Chapter 8

DISCUSSION

The hypothesis is that dextran is an essential factor in dental caries. Experimental evidence has been submitted which will permit the choice of a tenable position on this hypothesis.

Failure of dextran-fed, microbe-bearing Sprague-Dawley rats to experience dental caries disputed the concept that high molecular weight dextran present in the oral cavity is essential for dental caries development. However, this failure did not disprove the possibility that dextran is contributory or essential to dental caries induction. Several alternative explanations can account for this apparent contradiction:

1. The fluoride present in the tap water may have given sufficient protection to have inhibited caries development.

2. The structure (branching or chain length) of the commercial dextran may have rendered it non-adherent, although a 5% water suspension of this material was quite viscous. Gibbons and Nygaard (1968) have suggested that the quality of the dextran may influence its physical properties significantly.

3. A dextran-synthesizing organism may be necessary to get an agglutination-like response (Gibbons and Fitzgerald, 1969) to form an adherent tooth plaque.

4. A bacterial or a rodent, oral alpha 1,6' glucosidase might have degraded the dextran preventing dental accumulation and making glu-
cose available for growth. This possibility has human implications. Do caries-free humans possess oral \( \text{alpha} 1,6' \) glucosidases?

Although this experiment did not prove or disprove the essential nature of dextran in the dental caries process, it suggested some hesitance in accepting the idea that any dextran present in the rat oral cavity will facilitate bacterial colonization of the teeth with resulting tooth decay.

The cariogenic \textit{Lactobacillus casei} 4646 lacks extracellular enzymes including a dextranucrase which release glucose or fructose from sucrose. This caries inducing organism does not synthesize extracellular dextran when grown in a sucrose-containing environment. \textit{L. casei} 4646 does not exhibit an \textit{in vitro}, adherent growth ability. These results were consistent with the finding of Rosen, Lenney, and O'Malley (1968) that \textit{L. casei} 4646 formed no dental plaque when introduced as a monoinfecting agent in sucrose-fed gnotobiotic rats. Also these results supported the position that a bacterial organism could be caries inductive without synthesizing an adhesive, extracellular dextran which facilitated its adherent growth ability.

A possible objection to the \textit{Lactobacillus casei} 4646 work should be noted here. One cariogenic streptococcal strain has been found to have lost its cariogenicity after several years of laboratory subculturing (Gibbons and Fitzgerald, 1969). There is the possibility that the \textit{L. casei} 4646 cells which were used for the biochemical studies were not cariogenic.

Is there any additional evidence supporting the view that dextran is unessential for caries development and that dextranase would be an inadequate anticaries agent?
Two strains of microorganisms, *Odontomyces viscosus* (Jordan and Keyes, 1964, Howell et al., 1965, and Howell and Jordan, 1967) and *Streptococcus SS2* (Gibbons and Banghart, 1968), have been isolated and shown to synthesize exclusively levan from sucrose. These organisms induced carious lesions in the molars of sucrose-fed hamsters and rats respectively. These findings have not violated the concept that an extracellular polysaccharide (in these instances levan) was responsible for tooth colonization and thereby tooth decay. The only cariogenic organism examined and found lacking the ability to produce some detectable extracellular polysaccharide from sucrose has been *Lactobacillus casei* 4646. Table 6 lists the cariogenic organisms examined and found capable of utilizing sucrose to synthesize extracellular dextran and/or levan. These extracellular sucrose-utilizing, polysaccharide-synthesizing, cariogenic bacteria represented approximately 50% of the known cariogenic organisms.

A qualification should be introduced here concerning the observation that *Lactobacillus casei* 4646 has no dextran synthesizing ability yet is cariogenic. *L. casei* 4646 induced exclusively pit and fissure (crevice) carious lesions in the molars of sucrose-fed gnotobiotic rats (Rosen, Lenney, and O'Malley, 1968). Many of the dextran-synthesizing bacteria listed in table 4 induced smooth surface cavities in addition to pit and fissure cavities. For example, *Streptococcus SBEL* was found capable of inducing "extensive caries involving both fissures and smooth surfaces" in sucrose-fed gnotobiotic rats (Gibbons and Banghart, 1968). Keyes (1968b) has summarized this concept by writing:

... three different types of carious lesions affect teeth:
Table 6

Extracellular Polysaccharides Produced from Sucrose by Cariogenic Bacteria

<table>
<thead>
<tr>
<th>Genus</th>
<th>species</th>
<th>strain number</th>
<th>Extracellular Polysaccharide</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus</td>
<td></td>
<td>FA1</td>
<td>Dextran and levan</td>
<td>Wood and Critchley, 1966</td>
</tr>
<tr>
<td>Streptococcus</td>
<td>sanguis</td>
<td></td>
<td>Dextran</td>
<td>Carlsson and Krasse, 1968</td>
</tr>
<tr>
<td>Streptococcus</td>
<td></td>
<td>Ingbritt</td>
<td>Glucan and fructan</td>
<td>Dahlquist, Krasse, Olsson, and Gardell, 1967</td>
</tr>
<tr>
<td>Streptococcus</td>
<td></td>
<td>Mats</td>
<td>Glucan and fructan</td>
<td></td>
</tr>
<tr>
<td>Streptococcus</td>
<td></td>
<td>3720</td>
<td>Glucan and fructan</td>
<td></td>
</tr>
<tr>
<td>Streptococcus</td>
<td></td>
<td>GS5</td>
<td>Dextran</td>
<td>Gibbons and Banghart, 1967</td>
</tr>
<tr>
<td>Streptococcus</td>
<td></td>
<td>PK1</td>
<td>Dextran</td>
<td></td>
</tr>
<tr>
<td>Streptococcus</td>
<td></td>
<td>120</td>
<td>Dextran</td>
<td></td>
</tr>
<tr>
<td>Streptococcus</td>
<td></td>
<td>GF71</td>
<td>Dextran</td>
<td>Gibbons and Nygaard, 1968</td>
</tr>
<tr>
<td>Streptococcus</td>
<td></td>
<td>E49</td>
<td>Dextran</td>
<td>Fitzgerald, Spinell and Stoudt, 1968</td>
</tr>
<tr>
<td>Streptococcus</td>
<td></td>
<td>HS6</td>
<td>Dextran</td>
<td></td>
</tr>
<tr>
<td>Streptococcus</td>
<td></td>
<td>LM7</td>
<td>Dextran</td>
<td></td>
</tr>
<tr>
<td>Streptococcus</td>
<td></td>
<td>SL1</td>
<td>Dextran</td>
<td></td>
</tr>
<tr>
<td>Streptococcus</td>
<td></td>
<td>OMZ61E</td>
<td>Dextran</td>
<td>Guggenheim and Schroeder, 1967</td>
</tr>
<tr>
<td>Streptococcus</td>
<td></td>
<td>OMZ176E</td>
<td>Dextran</td>
<td>Gibbons and Loesche, 1967</td>
</tr>
<tr>
<td>Streptococcus</td>
<td></td>
<td>130</td>
<td>Dextran</td>
<td></td>
</tr>
<tr>
<td>Streptococcus</td>
<td></td>
<td>98E</td>
<td>Dextran</td>
<td></td>
</tr>
<tr>
<td>Streptococcus</td>
<td></td>
<td>OMZ71R</td>
<td>Dextran</td>
<td>König and Guggenheim, 1968</td>
</tr>
</tbody>
</table>
Table 6 (continued)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain/Type</th>
<th>Carbohydrate</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptococcus</td>
<td>OMZ51</td>
<td>Dextran</td>
<td>Bowen, 1968</td>
</tr>
<tr>
<td>Streptococcus</td>
<td>salivarius-SS2</td>
<td>Levan</td>
<td>Gibbons and Banghart, 1968</td>
</tr>
<tr>
<td>Odontomyces</td>
<td>viscous</td>
<td>Levan</td>
<td>Howell and Jordan, 1967</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>acidophilus-108TR</td>
<td>Dextran</td>
<td>Gibbons and Banghart, 1967</td>
</tr>
</tbody>
</table>

*Organisms enumerated here represent approximately 50% of the known cariogenic organisms. See Nolte (1968) for a more complete listing of cariogenic organisms.*
(1) those in crevices, (2) those on smooth surfaces, and (3) those on root surfaces. The interaction between bacteria and oral substrates may differ in each case. Lesions in crevices do not necessarily depend on adherent plaques, but other types do. In crevices the impaction and retention of food particles is important. On smooth surfaces and root surfaces the retention of adhesive microorganisms is essential. In the latter case it appears that the tenacity of microorganisms is more important than the tenacity of food residues.

However, there are a number of reasons that make the Lactobacillus casei 4646 observation and comparison to Streptococcus FA1 important:

a. Pit and fissure (crevice) cavities are frequently experienced by humans (Dirks, 1961). Therefore, any insight in crevice decay could be important in effectively controlling this aspect of the dental caries problem.

b. Burnett and Scherp (1968) have written that "very few lactobacilli were found on soft tissue; most were localized on the tooth surface most susceptible to dental caries." These authors continued that "more recent investigations, however, showed that lactobacillus-type organisms were present in almost every stage of caries in either enamel or dentin."

c. Streptococcus FA1, a dextran synthesizer, induced exclusively pit and fissure cavities in sucrose-fed gnotobiotic rats (Fitzgerald and Jordan, 1968). This fact reinforced the validity of comparing Lactobacillus casei 4646 and Streptococcus FA1.

2. The literature yielded additional evidence supporting the concept that dextran synthesis is not essential to dental caries induction. Tube-fed rats had no dietary nutrients orally and experienced no decay. The same sucrose-containing diet fed orally resulted in the development of plaque and carious lesions. However,
the tube-fed rats developed adherent, microbial accumulations on their molars (Kite, Shaw, and Sognnaes, 1950). This observation has led to the following conclusions:

a. Dietary sucrose is not essential for bacterial tooth colonization and dental plaque formation.

b. Dextran-synthesizing (or levan-synthesizing) organisms are not essential to the formation of visible, adherent tooth accumulation (assuming sucrose is the only substrate giving rise to dextran and levan).

c. Microbial accumulations on the teeth do not necessarily lead to the development of carious lesions.

d. Dextranase treatment probably would not have reduced or prevented the above described dental accumulation.

3. Fitzgerald (1968) confirmed and advanced point 2c above by writing that "three dextran producing strains of S. sanguis and 3 levan producing strains of S. salivarius were found to be incapable of inducing caries in the present study in spite of the fact that all strains caused the accumulation of plaque deposits in the infected rats." Fitzgerald concluded that "while the ability to produce extracellular polysaccharides seems to be a factor of importance in the bacterial etiology of caries it should be pointed out that neither dextran or levan production per se confer cariogenic potential on an organism." The observation that the cariogenic Lactobacillus casei 4646 has no extracellular enzymes including a dextranucrase which release glucose or fructose from sucrose (Grigsby and Kline, 1969) was consistent with Fitzgerald's work. The L. casei 4646 observation is the stronger argument against the essentiality of dextran
in the dental caries process. An objection to Fitzgerald's finding is that these dextran and levan producing, non-cariogenic streptococcal organisms might be cariogenic under different conditions (although this is doubted). This situation is similar to conditions observed clinically in the human system. Many human teeth accumulate visible, adherent plaque but without carious lesions while other human teeth experience caries without a demonstrable adherent plaque (Miller, 1902).

4. Another argument against an essential nature for dextran in the caries process was provided by Rosen (1969), if sucrose is assumed to be the only substrate for dextransucrase in dextran synthesis (Neely, 1960 and Ebert and Schenk, 1968). Rosen found that glucose-fed gnotobiotic rats monoinfected with *Streptococcus FA1* or *Lactobacillus casei* 4646 experienced tooth decay. Other investigators (Edwardsson and Krasse, 1967, and Frostell, Keyes, and Larson, 1967, and König, 1968) have substantiated the observation that diets containing carbohydrates other than sucrose have potentiated cariogenic microorganisms resulting in tooth decay in hamsters and rats. Carbohydrates tested and found caries conducive were glucose, fructose, combinations of glucose and fructose, hydrogenated starch, lactose, and maltose.

However, this line of reasoning must be considered within the framework of some additional evidence:

a. When mixed bacterial cultures obtained from dental plaque on rat molars were grown in a medium containing one of the

$^2$Hydrogenated starch = hydrogenated potato starch contained sorbitol and dextrins 2-10 glucose units long with a sorbitol residue at one end of the chain (product 6563 Lyckeby Starch Refining Co.).
following carbohydrates, sucrose, glucose, or maltose, the extracellular slime which was produced reacted with antidextran antiserum (Shaw, Krumins, and Gibbons, 1967).

b. The polysaccharides isolated from dental plaque on the molars of rats fed diets containing one of the following carbohydrates, sucrose, corn starch, glucose, fructose, or maltose, were composed of predominantly glucose and all yielded glycerol after Smith degradation (Leach et al., 1969).

c. Leach (1969) has concluded: "It is thus apparent that the formation of dextran by the oral organisms is not exclusive to sucrose as implied from the previous observations. The dextrans produced in the presence of these other sugars could not have arisen from the action of the enzymes described earlier as this still remains energetically impossible. Some other mechanisms must be operative but these are not remotely understood at the present time and much further work will be required before this can be so."

5. Finally, dextranase was ineffective in preventing dental caries, pit and fissure and smooth surface, in sucrose-fed rats with a complex, uncontrolled microbial flora (Köning and Guggenheim, 1968 and Guggenheim, König, Mühlemann, and Regolati, 1969). Guggenheim, König, Mühlemann and Regolati, (1969) concluded that "there are other acidogenic bacteria the cariogenicity of which would not be decreased by the administration of dextranase."

The above five points with the experimental evidence advanced in this thesis have indicated that extracellular dextran synthesized from sucrose is probably not a fundamental component in the dental caries process. Therefore, the property of dextran synthesis from
sucrose is not the unifying hypothesis which dental investigators have sought. However, this conclusion must not be construed as invalidating dextran synthesis as a mechanism facilitating tooth colonization by bacteria. On the contrary, dextran synthesis is the first molecular-level explanation offered by oral biologists to account for active bacterial implantation on teeth.

In summary, the main points cited in this thesis to support the view that dextran synthesis is unessential to the dental caries process are presented below:


2. Lactobacillus casei 4646 has no extracellular dextranucrase enzyme or other extracellular enzymes which release glucose or fructose from sucrose.

3. L. casei 4646 does not synthesize extracellular polysaccharide of the dextran class when grown on sucrose-containing medium.

4. L. casei 4646 does not exhibit in vitro, adherent growth on stainless-steel wire when grown on sucrose-containing medium.

5. L. casei 4646 does not cause dental plaque formation on the molars of sucrose-fed gnotobiotic rats (Rosen, Lenney, and O'Malley, 1968).

6. L. casei 4646 induces dental caries of the pit and fissure class when introduced as a monoinfecting agent in sucrose-fed gnotobiotic rats (Rosen, Lenney, and O'Malley, 1968).

These observations advocated rejection of the hypothesis that extracellular dextran is an absolute requirement in the dental caries
process. Therefore, dextranase will be an effective anticaries agent only against organisms such as *Streptococcus E-49* (Fitzgerald, Keyes, Stoudt, and Spinell, 1968) which depend on the synthesis of dextran from sucrose for tooth colonization. This position predicts that dextranase treatment of sucrose-fed gnotobiotic rats infected with *Lactobacillus casei* 4646 would have no effect on the rats' caries experience.
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organism isolated from periodontal plaque in hamsters.

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