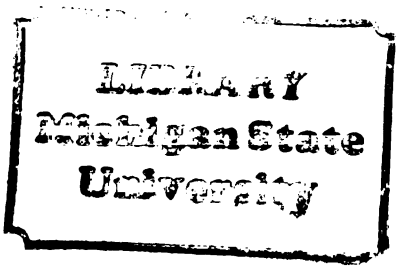

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AND DISTRIBUTION OF GLUCOSYLTRANSFERASE

IN STREPTOCOCCUS MUTANS 6715

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THE EFFECT OF SURFACTANTS UPON THE ACTIVITY AND DISTRIBUTION OF GLUCOSYLTRANSFERASE IN *STREPTOCOCCUS MUTANS* 6715

By

Catherine Wernette

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ABSTRACT

THE EFFECT OF SURFACTANTS UPON THE ACTIVITY
AND DISTRIBUTION OF GLUCOSYLTRANSFERASE
IN STREPTOCOCCUS MUTANS 6715

By

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A modified Somogyi-Nelson procedure was used to determine the amount of reducing sugar produced during the reaction of Streptococcus mutans 6715 glucosyltransferase (GTF) with sucrose in the presence of the surfactants glycerol monolaurin (GML) and sodium lauryl sulfate (SLS). GML and SLS both reduced extracellular culture filtrate GTF enzyme activity. Secondly, Streptococcus mutans 6715 cultures were grown in medium containing GML, Tween 80, or SLS. Whole culture GTF activity was decreased when the organism was grown in the presence of GML. Tween 80 caused a large increase in whole culture GTF activity, whereas SLS resulted in no change in the GTF activity as compared to the control.

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INTRODUCTION

Fats, which compose 30-40% of the human diet, have been examined for their antimicrobial effects and may be useful as non-toxic prophylactic agents in the prevention of dental caries. Several studies have demonstrated that the development of dental caries can be retarded by fats. Lard (Roseburg, et al., 1939), butterfat (Constant, et al., 1954), corn oil (Roseburg, et al., 1939), and margarine (Wynn, et al., 1960) have all been shown to inhibit the development of animal caries.

The antimicrobial properties of fatty acids are well known (Bayliss, 1936; Kodicek, 1949). Kabara et al. (1972) examined 30 purified fatty acids and derivatives for antimicrobial activity against 12 gram-positive organisms (including Streptococcus mutans 6715) and 8 gram-negative organisms. Of the saturated fatty acids the average minimal inhibitory concentration (MIC) decreased from the highest value for the C_6 and C_{20} fatty acids to the lowest for the C_{12} (lauric acid). Addition of a cis double bond in the delta 9 position increased the activity of the unsaturated C_{14}, C_{16} and C_{18} fatty acids. Addition of a second double bond further increased the bacteriostasis of the C_{18} fatty acid.

Since lauric acid, C_{12}, had the greatest antimicrobial activity of the saturated fatty acids, the effect of esterification of lauric acid with various alcohols was examined. Lauryl alcohol and lauryl aldehyde were similar in activity to the free acid. Lauric acid esterified with

cholesterol or methanol showed no inhibition. The diglyceride (1,3-dilaurin) and triglyceride (trilaurin) were less active than the fatty acid. The monoglyceride (1-mono-laurin) was found to be more active than the free acid.

While screening some 40 natural or synthetic lipophilic compounds for antimicrobial activity, Kabara et al. (1977) found that 1-mono-laurin was the best antimicrobial, particularly against gram-positive microorganisms.

Because most cariogenic bacteria are gram-positive microorganisms and since monolaurin is generally recognized as safe (GRAS) by the Food and Drug Administration, monolaurin was used in an *in vivo* study to determine its effects upon the development of dental caries in Osborne-Mendel rats (Kabara, et al., 1979). Rats were fed the modified high sucrose NIH diet 2000 (Keyes, 1959) containing either 2% Crisco^{R} (control) or 2% Lauricidin^{R} (a 90% pure preparation of the monoglyceride of lauric acid). The animals were orally inoculated with *Streptococcus mutans* 6715 and maintained on the diets for four weeks. At the end of the experimental period the animals were killed and their teeth were evaluated for both microorganisms and dental caries. Due to wide variations in oral microbial populations among animals, no statistical differences were observed between the Crisco^{R} and Lauricidin^{R} groups. However, a statistically significant reduction in smooth surface dental caries was measured in rats fed Lauricidin^{R} (Schemmel, et al., 1979).

The antibacterial effect of the surfactant, Lauricidin^{R}, may play a role in the inhibition of dental caries, but other mechanisms may also contribute to the observed reduction in smooth surface dental caries.

One of the inhibiting abilities of surface active compounds has been shown to be upon the activity of the glucosyltransferase (GTF) enzyme of many of the cariogenic streptococci, notably, Streptococcus mutans (Christiansen and Kilian, 1975).

The purpose of this investigation is to compare the effects of the surfactants sodium dodecyl sulfate (SDS), Tween 80 and glycerol monolaurin upon the activity and distribution of glucosyltransferase in Streptococcus mutans 6715.

LITERATURE REVIEW

Dental Caries

Dental caries is a unique disease in many respects. It appears to be a product of civilization because its incidence increases with the standard of living (Bibby, 1978). The disease progresses with no systemic response, such as inflammation or fever, and no repair mechanisms are initiated. Development of dental caries is affected by the character of food, bacteria, saliva and tooth structure. Almost 100% of the population requires treatment which must be provided by trained, well-paid technicians who need expensive materials and equipment. To be effective, preventive measures must demand little trouble or sacrifice from the public.

In the 1890's, W.D. Miller incubated a tooth in a mixture of saliva and bread and observed that the salivary bacteria fermented the carbohydrate and produced sufficient acid to decalcify the tooth (Miller, 1890). As a result, Miller proposed that bacteria were the etiologic agents of dental caries (Harris, 1968). Dental caries has since been defined as the localized destruction of tooth tissue by bacterial action (Gibbons and van Houte, 1975). Germ-free animals do not develop dental caries (Orland, et al., 1954) and antibiotics prevent progression of the disease (Shaw, 1959).

The temperature (35-36^{o} C), availability of nutrients and

differences in oxygen tension found in the oral cavity make it a suitable habitat for many bacterial types. The predominant organisms identified in dental plaque are facultative streptococci, facultative diphtheroids, peptostreptococci, and small percentages of Veillonella, Bacteroides, Fusobacteria, Neisseria and Vibrio species (Nolte, 1977). These organisms can gain access to the oral cavity by water, food, air or hands.

The first evidence of dental plaque, and the initiation of dental disease, is the development of the enamel pellicle which consists of acidic proteins and glycoproteins from saliva which adsorb to the hydroxyapatite of the tooth surface. Microorganisms colonize the pellicle and their production of extracellular polysaccharides causes increased plaque volume and adherence (Frostell and Ericsson, 1978).

Sucrose or other easily fermentable carbohydrates are required to initiate dental caries (Newbrun, 1967; Marthaler, 1967). The cariogenic streptococci produce the extracellular polysaccharides dextran, mutan and levan from sucrose by means of the enzymes glucosyl- and fructosyl-transferase. The ability to form extracellular polysaccharides, and consequently enhanced plaque production, is considered to be one of the main explanations for the cariogenicity of Streptococcus mutans (Fitzgerald, 1976). An intracellular polysaccharide of Streptococcus mutans, glycogen, and the extracellular levan can be utilized as sources of energy, when exogenous sucrose or other simple sugars are absent, resulting in the production of acid. While extracellular glucan (dextran or mutan) may be used as a carbon source, strong acid

production is unlikely to occur (Huis in't Veld and Dirks, 1978). Addition of exogenous fermentable sugar to dental plaque results in rapid acid formation. Acid production by these organisms causes demineralization of the enamel surface of the tooth and initiates a caries lesion.

Possible Preventive Measures

Restriction of sugar intake, use of sugar substitutes, alteration of the normal oral flora, immunization and treatments making use of enzyme inhibitors, antibiotics, hydrolytic enzymes or antiseptics may all potentially inhibit development of dental caries.

Restriction of sugar from the diet would substantially reduce the incidence of dental caries; however, it is unlikely that people would voluntarily alter their diets. An attractive alternative is the use of sugar substitutes, but relatively few human clinical trials have been carried out to determine their effects on the incidence of dental caries.

In order to be effective the sugar substitute has to have sweetness, palatability, chewability and solubility similar to conventional sweets (Newbrun and Frostell, 1978). Lycasin, a hydrogenated potato starch hydrolysate, has been used in human studies in Sweden and its effect on caries development compared with that of sucrose. No definitive statistical differences were observed.

Sorbitol has been shown to reduce the incidence of caries and reduce plaque accumulation in rodents, but this was accompanied by decreased food intake and weight gains so its effects have been

questioned (Newbrun and Frostell, 1978). The addition of sorbitol, arabinatol, ribitol or mannitol to saliva in the presence of plaque bacteria all produced decreased plaque pH because of acid production (Hayes and Roberts, 1978).

Xylitol has demonstrated very low cariogenicity in human studies and no pH decrease is observed when it is added to mixtures of saliva and plaque bacteria (Hayes and Roberts, 1978). However, the reduction in caries may have been due to remineralization of early lesions once the use of sucrose had been discontinued (Scheinin and Makinen, 1975). Xylitol usage has resulted in some gastrointestinal side effects and its cost is approximately ten times greater than that of sucrose; therefore, the benefits of xylitol usage are in doubt.

Implantation of organisms not normally present in the oral cavity to alter the microbial balance has been attempted but the organisms are rapidly eliminated. To establish new organisms the diet and salivary compositions would probably also have to be changed (Frostell and Ericsson, 1978). Another alternative is to introduce non-pathogenic Streptococcus mutans into the oral cavity. Dental caries may be decreased due to the decreased cariogenicity of the competing microorganisms (deStoppelaar, Konig, Plasschaert and Van der Hoeven, 1971).

Immunization against dental caries has been attempted but it is not possible to utilize a specific antibody reaction against the mixed oral flora. Vaccines developed against glucosyltransferase and fructosyltransferase have failed to protect monkeys from the development of dental caries (Bowen, Cohen and Colman, 1975) and vaccines against

dextrans and mutans have shown no effect.

Enzyme inhibitors have been examined as potential therapeutic agents; however, most have general activity and are too toxic or they have specific activities and are too ineffective for clinical use. Sodium lauroyl sarcosinate and sodium dehydroacetate have been used in toothpastes but evidence for inhibitory effects on dental caries development is conflicting (Frostell and Ericsson, 1978). Fluoride inhibits bacterial growth, polysaccharide production and/or acid production (Hamilton, 1977; Rolla, 1977). High fluoride concentrations are required before acid production is reduced. Fluoride may reduce plaque formation by reduction of the free surface energy of the tooth (Rolla and Melsen, 1975); however, bacteria may acquire resistance to fluoride by reducing membrane permeability (Frostell and Ericsson, 1978).

The antibiotics penicillin, aureomycin, terramycin, streptomycin, bacitracin, subtilin, chloromycetin, concanavalin A, polymixin and others have been shown to reduce plaque and caries (Loesche, 1975). The development of drug resistant bacteria and toxic or allergic reactions of patients become problems upon continued usage.

Hydrolytic enzymes such as pronase, neuraminidases and other bacterial hydrolases are not effective in dissolving dental plaque. Proteolytic enzymes such as trypsin and pepsin do not dissolve plaque and may damage oral soft tissues. Enzymes which attach the alpha (1,6)- and alpha-(1,3)-linkages of extracellular polysaccharides may inhibit dental plaque formation, but do not remove established plaque. The use of enzymes in caries prevention remains open to future investigation (Frostell and Ericsson, 1978).

After rinsing or brushing with antiseptics the bacterial counts of the oral cavity are reduced 50%, but the normal flora regenerates in approximately two hours due to the short generation times (about 20 minutes) of many of the oral streptococci (Loesche, 1975). Antiseptics are not generally practical for everyday use. Quaternary ammonium compounds reduce plaque flora to 10-30% of the normal population. Oxidizing agents such as hydrogen peroxide and sodium perborate reduce plaque accumulation but cannot be used frequently.

The surfactants chlorhexidine, sodium dodecyl sulfate and sodium deoxycholate have also been shown to reduce plaque, caries and periodontal disease (Loesche, 1975; Christiansen and Kilian, 1975) but, again, they cannot be generally used due to the possible development of bacterial drug resistance (Frostell and Ericsson, 1978). The patient may develop toxic or allergic reactions with prolonged usage and taste sensations are often affected.

General Characteristics of *Streptococcus mutans*

Clarke (1924) originally isolated a group of nonhemolytic streptococci from carious human teeth which he called *Streptococcus mutans*. Many characteristics of the strains isolated served to distinguish them from other streptococci. All of the strains fermented mannitol, sorbitol, inulin, raffinose, salicin, lactose and melibiose. Edwardson (1967) found that these strains formed dextran from sucrose, hydrolyzed esculin, grew in 4% NaCl broth and 10% bile agar.

The glucosyl- and fructosyl-transferase enzymes produced by these organisms utilize the energy contained in the disaccharide bond of sucrose

for polymerization of glucan and fructan (Gibbons and van Houte, 1975). Dextranase is produced by many strains of Streptococcus mutans (Staat and Schachtele, 1974). The enzyme may function as a means of using extracellular soluble dextran during periods of nutritional deprivation or it may be involved in the formation of highly branched dextrans by hydrolyzing soluble dextrans to oligosaccharides which could be incorporated by glucosyltransferase as a branch. Invertase-like enzymes are also produced by these organisms and may be the main factor involved in the decomposition of sucrose in dental plaque (Birkhed and Frostell, 1978).

Streptococcus mutans was found to be serologically heterogeneous. Bratthall (1970) described five type antigens which were designated a, b, c, d and e. Types a and b were purified and identified as polysaccharides present in or on the bacterial cell wall (Mukasa and Slade, 1973). Type a has major components of rhamnose, galactose and glucose (Bleiweis, Craig and Zinner, 1971). The serological specificity of the type a determinant has been related to a terminal diglucose (Mukasa and Slade, 1973). Type b strains of Streptococcus mutans have a distinct pattern of cell wall carbohydrates. The major sugars present are rhamnose and galactose (Bleiweis, Craig and Zinner, 1971). Two type b antigens have been isolated and characterized from Streptococcus mutans strain FA1 (Mukasa and Slade, 1973). Type b, II, lacks rhamnose and contains 39% protein. The antigenic specificity is related to galactose, rhamnose and protein acting as carriers for the glucose polymers. Two type c antigens were isolated from Streptococcus mutans strain Ingbritt (Linzer, Gill and Slade, 1976). Both were

polysaccharides composed of rhamnose and glucose in a ratio of 2.4:1. They differed in minor components, phosphorus and protein. The serological specificity of the c antigen is related to a terminal glucose. The type d antigen is a polysaccharide composed of galactose and glucose in a 2:1 ratio (Linzer and Slade, 1974). The antigen has two serologically active sites. One site is specific for group d and a second site is common to both group d and group a strains. The antigenic specificity of type d strains depends on a terminal D-galactose. The composition of the type e antigen has not been described at this time, but is similar to type c in that its major carbohydrates are rhamnose and glucose. Two new serotypes have been proposed (Perch, Kjems and Ravn, 1974) and designated types f and g. Type f has the same carbohydrate pattern as types c and e. Type g has the same carbohydrate pattern as types a and d. Both f and g cross react with their particular type mates.

As may be expected, Streptococcus mutans strains have been found to be genetically heterogeneous (Coykendall, 1974). It was determined by genetic, antigenic and biochemical tests that the organisms commonly identified as Streptococcus mutans from human dental plaque are composed of four different species of streptococci. The characteristic colony (on media containing sucrose), polymer production and mannitol and sorbitol fermentation define the "mutans-group" of streptococci (Coykendall and Lizotte, 1978). The species Streptococcus mutans has a DNA base content (mole percent G+C) ranging from 36-38, does not produce ammonia from arginine, is resistant to bacitracin and ferments raffinose. The DNA base content of Streptococcus rattus ranges from

41-43 and the organism produces ammonia from arginine. Streptococcus cricetus has a DNA base content of 42-44 and is sensitive to bacitracin. Streptococcus sobrinus has a DNA base content of 44-46 and the organism does not ferment raffinose.

Streptococcus mutans was the predominant Streptococcus mutans-like organism isolated from dental plaque samples tested.

Extracellular Polysaccharides

Human and rodent cariogenic streptococci produce large quantities of extracellular carbohydrate from sucrose, whereas non-cariogenic bacteria fail to do so (Gibbons, et al., 1966). Extracellular polysaccharide synthesized by cariogenic bacteria was shown to be present as a component of the matrix of human dental plaque (Gibbons and Banghart, 1967). At least one fraction of the polysaccharide formed by the cariogenic streptococcus FA-1 was found to be polyglucose with 1-6 and/or 1-2 hexopyranoside linkages confirming the material to be dextran (Wood and Critchley, 1966). Thin layer and paper chromatography of acid hydrolysates of the polysaccharide produced by streptococcus strain GS-5 indicate it was primarily a glucose polymer containing small amounts of fructose and was rapidly hydrolyzed by the dextranase activity of the culture filtrates of Penicillium funicolosum (N.R.R.L. 1768) (Gibbons, et al., 1966). Dextran (glucan) precipitated by ethanol from the culture medium of Streptococcus mutans E-49 was found to be highly branched, a glycogen-like structure, with a linear (1, 6)-backbone and (1,3)- branches (Lewicki, et al., 1971).

Water insoluble glucan isolated from culture filtrates of Streptococcus mutans strain Ingbritt A represented about 25% of the total glucan and contained both alpha-(1,6)- and alpha-(1,3)-linkages. Water soluble glucan contained principally alpha-(1,6)-linkages (Baird, Longyear and Ellwood, 1973). The absence of virulence observed in mutants is probably caused by a failure to form the alpha-(1,3) rich component (Freedman, Birked and Granath, 1978). Four types of glucan produced by Streptococcus mutans have been isolated, three of which are water soluble. The water-insoluble and one of the water-soluble glucans have molecular weights of 15×10^6, are mostly alpha-(1,3)-linked glucose and are resistant to dextranase. The insoluble glucan may form by aggregation of high molecular weight water-soluble glucan. The second water-soluble glucan isolated had a molecular weight ranging from 3×10^5 to 5×10^6, was highly branched, and resistant to dextranase. The last soluble glucan was dextranase sensitive, consisted of mostly alpha-(1,6)-linked glucose in a linear arrangement, and had a molecular weight of 10^4 or less (Inoue and Koga, 1979).

Glucosyltransferase (Dextransucrase)

Wood (1967) described the isolation of an enzyme responsible for the synthesis of dextran from sucrose by streptococcus FA-1. The activity of the enzyme, dextransucrase, was assayed by estimating the release of reducing sugar from sucrose using a modification of the Somogyi method (Marais, et al., 1966). Optimal activity of the enzyme was between pH 5.2 and 7.0, at $45^{\circ}C$ (Carlsson, Newbrun and Krasse, 1969).

No polysaccharide was produced from maltose, lactose, fructose, melibiose, galactose, glucose, raffinose, trehalose, cellibiose, melezitose, alpha-methyl glucoside, sucrose monophosphate or sucrose diphosphate (Carlsson, et al., 1969; Newbrun and Carlsson, 1969).

Fructose was found to be directly inhibitory for the enzyme (Gibbons and Nygaard, 1968). Urea and sodium dodecyl sulfate completely inhibited activity (Chludzinski, Germaine and Schachtele, 1974). Dextran was found to stimulate activity of the enzyme (Germaine, Chludzinski and Schachtele, 1974).

Glucosyltransferase activity was almost totally extracellular when Streptococcus mutans strain 6715 was grown in brain heart infusion broth or trypticase glucose broth. When grown in trypticase soy broth, 40% of the total glucosyltransferase activity was cell-associated, due to trace amounts of sucrose in the soy extract (Spinell and Gibbons, 1974).

The distribution of glucosyltransferase activity (extracellular to cell-associated) changes depending on the medium used. Media free of detectable contaminating sucrose favors extracellular enzyme activity (Janda and Kuramitsu, 1976). Therefore, it has been suggested that the distribution of glucosyltransferase activity can be regulated independently of its synthesis (Montville, Cooney and Sinskey, 1977).

The addition of the surfactant Tween 80 to the growth medium resulted in increased levels of glucosyltransferase. However, Tween 80 did not directly affect the glucosyltransferase activity (Umesaki, Kawai and Mutai, 1977). Penicillin treatment of Streptococcus mutans enhanced the levels of extracellular glucosyltransferase activity

(Janda and Kuramitsu, 1978) as did addition of linoleic or lauric acid to culture broth (McChesney, et al., 1978). Mono- or divalent cations increased both total glucosyltransferase activity and production of water-insoluble glucans (Mukasa, et al., 1979).

MATERIALS

Bacterial Strain

Streptococcus mutans 6715, a cariogenic organism first described by Fitzgerald, et al., (1968), was obtained from Dr. Rachel Larson, National Institute of Dental Research, Bethesda, Maryland, U.S.A.

Growth of Bacterial Cultures

The liquid growth medium for the bacterial cultures was Trypticase Soy Broth, pH 7.2, and the solid medium was Trypticase Soy Agar, both from Baltimore Biological Laboratories of Cockeysville, Maryland. The surfactants used were Tween 80, sodium lauryl sulfate (both from Sigma Chemical Co., St. Louis, Missouri) and glycerol monolaurin (Lauricidin^{R}, obtained from Med-Chem Laboratories, Monroe, Michigan). Turbidity of growth was measured using a Bausch and Lomb Spectronic 20.

Preparation of Cultures for Glucosyltransferase Assay

The 24-hour bacterial cultures were centrifuged using a Model HN-S Centrifuge, International Equipment Company, Needham Heights, Massachusetts.

Glucosyltransferase Assay

Dextran T-10 was obtained from Pharmacia, Inc. of Uppsala, Sweden. Sucrose was from Mallinckrodt Laboratory Chemicals of St. Louis, Missouri. Sigma Chemical Co., also of St. Louis, was the source for sodium acetate, 0.3 N $(Ba(OH)_2$, $ZnSO_4$, arsenomolybdate and all chemicals used to prepare both the Somogyi reagent and Nelson's color reagent.

Glucose Oxidase Assay

The glucose diagnostic kit No. 510 from Sigma Chemical Co., St. Louis, Missouri, was used for the determination of glucose.

METHODS

Growth of Bacterial Cultures

The organism was grown in trypticase soy broth (BBL, Cockeysville, Maryland, U.S.A.). When required, Streptococcus mutans 6715 was grown in the same medium supplemented with 5, 10, or 15 µg/ml glycerol monolaurin (Lauricidin^{R}, Med-Chem Laboratories, Monroe, Michigan, U.S.A.); 10, 100, or 1,000 µg/ml Tween 80 (polyoxyethylene sorbitan monooleate, Sigma Chemical, St. Louis, Missouri, U.S.A.); or 15 µg/ml sodium lauryl sulfate (Sigma Chemical). The pH of the medium was adjusted to 7.2. Cultures were incubated at 37°C in the Gas-Pak system (BBL) for 24 hours. The cell populations were equalized by adjusting the optical density of all cultures to 0.5 at 550 nm (10^{6} CFU/ml).

Preparation of Glucosyltransferase Sources

The bacterial cells were harvested by centrifugation (10,000 x g). The culture supernatant was filtered using a 0.22 µm filter (Millipore Corporation, Bedford, Massachusetts, U.S.A.) and was either used immediately or stored frozen at -30°C. The cell-pellet, when required for use, was subjected to eight cycles of freeze-thaw using dry ice to break the cells and stored frozen. The cell-pellet was diluted to the same volume as the corresponding culture supernatant, 50 ml, prior to use.

Glucosyltransferase Assay

The glucosyltransferase assay was a modification of the methods of Christiansen and Kilian (1975) and Chludzinski, Germaine and Schachtele (1974). The reaction mixtures consisted of 100 mM sucrose, 20 µM dextran T-10, 250 mM sodium acetate buffer (pH 5.5), the appropriate concentration of surfactant (if required), and the crude glucosyltransferase preparation; either the culture filtrate (extracellular) glucosyltransferase or the cell-associated (cell-pellet) glucosyltransferase. The total volume of the reaction mixture was 5 ml. The reaction mixtures were incubated in shaker water bath at 37°C. The reactions were stopped by addition of 0.3 N $Ba(OH)_2$ and 5% $ZnSO_4$ (Nelson, 1944) and then filtered through Whatman No. 1 filter paper. Three ml of filtrate were added to 3 ml of Somogyi reagent (Somogyi, 1945; Somogyi, 1952; Marais, et al., 1966); and the test tubes containing the solutions were placed in a boiling water bath for 10 minutes, cooled, and 2 ml of Nelson's arsenomolybdate reagent (Nelson, 1944) were then added. The solutions were diluted to 50 ml in volumetric flasks. Color was allowed to develop, and the optical density at 750 nm was determined after 30 minutes.

A reagent blank contained 100 mM sucrose, 20 µM dextran T-10, 250 mM sodium acetate buffer and boiled (10 minutes) enzyme preparation. The blank was used to correct for any nonenzymatic activity present in the reaction mixtures. No growth inhibitor was included in the cell-associated enzyme assay system.

Glucose Oxidase Assay

The glucose oxidase method was used to determine the presence of free glucose in the enzyme reaction system. The glucose oxidase assay is specific for glucose and demonstrated that free glucose is either not liberated from sucrose, or is liberated at nondetectable levels under these conditions.

RESULTS

Addition of Surfactants to Glucosyltransferase Assay Mixture

Relatively low concentrations (5-15 µg/ml) of glycerol monolaurin (GML) or sodium lauryl sulfate (SLS) added to the glucosyltransferase (GTF) assay mixture caused a decrease (14-22%) in the crude culture filtrate GTF activity after a 2 hour interval (Table 1). Tween 80 could not be tested in this manner as a gelatinous precipitate formed during the Somogyi-Nelson procedure and interfered with subsequent determination. Using this method, higher concentrations of GML or SLS gave ambiguous results due to interference caused by their insolubility at these levels.

The Effect of Surfactants on Cell-Associated and Extracellular Culture Filtrate GTF Activity

S. mutans 6715 was grown in the presence of three surfactants to determine what effect they would have upon the relative amount of GTF produced and to determine the percentages of extracellular and cell-associated GTF. Bacteria were grown for 24 hours in the presence of 5, 10, 15 µg/ml; and the culture filtrates and cell-pellets were assayed for their GTF activity, as described earlier (Table 2). GML supplemented cultures contained significantly lower levels of culture filtrate GTF activity compared with controls. Cell-associated activity was

was variable but similar to control levels.

The bacteria grown in the presence of Tween 80 or SLS supplemented media were treated in the same manner (Table 3). Tween 80 added to the growth medium caused increased levels of GTF activity in both culture filtrate and cell-associated fractions. Tween 80 at 1,000 µg/ml raised the activity of GTF in whole culture to levels 110% higher than the control value.

Under these same conditions SLS at 15 µg/ml caused little or no change in either the amount of culture filtrate or cell-associated GTF activities.

TABLE 1. Effect of glycerol monolaurin (GML) and sodium lauryl sulfate (SLS) upon crude extracellular glucosyltransferase (GTF) of Streptococcus mutans 6715

Surfactant	Concentration (µg/ml)	GTF Activity	Activity of Control
Control	0	918±180	100
GML	5	792±108	86
	10	738± 72	80
	15	720± 18	78
SLS	15	774±126	84

The surfactants were added to the GTF assay to give the designated final concentrations. The culture filtrate was used as the source of GTF activity, which is reported as µg of reducing sugar (fructose) per ml of GTF assay mixture after 2 hours of incubation at 37°C. The activity is shown as the mean value of two trials, each in duplicate, ± the standard deviation.

TABLE 2. Glucosyltransferase activity in Streptococcus mutans 6715 cultures supplemented with glycerol monolaurin (GML)

Surfactant	Concentration (µg/ml)	Glucosyltransferase Activity		
		Culture Filtrate	Cell-Associated	Total
Control	0	1026± 23(100)	5238±432 (100)	6264±455 (100)
GML	5	684± 54(67)	4968±432 (95)	5652±486 (90)
	10	157±102(15)	5418±378 (103)	5580±379 (89)
	15	252± 90(24)	5634±139 (108)	5886±228 (94)

Streptococcus mutans 6715 cultures were grown in Trypticase soy broth, pH 7.2, containing the designated concentration of surfactant. Glucosyltransferase activity was determined after 2 hours of assay mixture incubation at 37°C as µg reducing sugar per ml of GTF assay mixture. The results are reported as culture filtrate (extracellular GTF), cell-associated (cell pellet GTF) and total (whole culture GTF) values. Each value is the mean of two trials, each in duplicate, the standard deviation. The numbers in parentheses represent percent activity of the control value.

TABLE 3. Glucosyltransferase activity in *Streptococcus mutans* 6715 cultures supplemented with Tween 80 or sodium lauryl sulfate (SLS)

Surfactant	Concentration (µg/ml)	Glucosyltransferase Activity		
		Culture Filtrate	Cell-Associated	Total
Control	0	1350±342 (100)	6138±576 (100)	7488±918 (100)
Tween 80	10	1350±414 (100)	7182±450 (117)	8532±864 (114)
	100	2898±198 (214)	6264±432 (102)	9162±630 (122)
	1000	5418±1026(401)	10368±1044(169)	15786±2070(210)
SLS	15	1368±468 (101)	6066±504 (99)	7434±972 (99)

Streptococcus mutans 6715 cultures were grown in Trypticase soy broth, pH 7.2, containing the designated concentration of surfactant. Glucosyltransferase activity was determined after 2 hours of assay mixture incubation at 37°C as µg reducing sugar per ml of GTF assay mixture. The results are reported as culture filtrate (extracellular GTF), cell-associated (cell pellet GTF) and total (whole culture (GTF) values. Each value is the mean of two trials, each in duplicate, ± the standard deviation. The numbers in parentheses represent percent activity of the control value.

DISCUSSION

Streptococcus mutans has been implicated as a causative agent in the development of smooth surface dental caries in laboratory animals and humans (Fitzgerald and Jordan, 1968; Fitzgerald, 1968; Guggenheim, 1968; Zinner and Jablon, 1968). Sucrose present in the diet has been shown to be metabolized by microorganisms on the tooth surface (Krasse, 1965; Wood and Critchley, 1966; Gibbons and Banghart, 1967) and can function as an energy source during bacterial growth or as a substrate for the glucosyltransferase enzyme(s) (GTF) produced by these organisms resulting in extracellular glucan formation (Guggenheim, 1968). Only a small portion of the sucrose is used for glucan synthesis. When carbohydrates limit growth, it has been shown that less than 15% of the glucose is polymerized (Robrish and Krichevsky, 1972). However, the glucans produced have been associated with the attachment of the microorganism to the tooth surface and the development of dental plaque.

Laboratory animals fed diets containing fats or oils have been shown to exhibit reduced incidence of dental caries (Constant, et al., 1954; Roseburg, et al., 1939; Wynn et al., 1960). Schemmel, et al. (1979) have demonstrated reduced smooth surface dental caries in rats fed diets containing 2% glycerol monolaurin. The mechanism of caries reduction is unknown; however, fats and oils may reduce adherence of food or protect tooth enamel from bacterial attack. McChesney, et al.

(1978) have postulated that fatty acids may decrease plaque formation, alter glucan binding by cells, or interfere with bacterial production of glucosyltransferase. It is possible that the monoglyceride, glycerol monolaurin, and certain other surfactants may exert their anticariogenic action in a similar manner.

This report examines two aspects of the effects of surfactants upon Streptococcus mutans 6715 and the glucosyltransferase produced by this strain of bacteria. The first objective was to measure the effect of each surfactant upon crude extracellular glucosyltransferase. The second objective was to determine the relative levels of extracellular and cell-associated glucosyltransferase from bacterial cultures grown in the presence of these surfactants. Determination of reducing sugar was accomplished by an adaptation of the Somogyi-Nelson technique (Nelson, 1944; Somogyi, 1945; Somogyi, 1952; Marais, et al., 1966) and was used as an indirect measure of glucan synthesis and, therefore, glucosyltransferase activity.

The GTF activity was measured by determining the amount of free fructose released during the GTF reaction with sucrose. In the reaction between GTF and sucrose, glucose and fructose are formed as reaction products. While the glucose is polymerized to form a growing glucan chain (Wood and Critchley, 1966; Lewicki, et al., 1971), the fructose becomes available as an energy source for the microorganism (Gibbons and Banghart, 1967; Robrish and Krichevsky, 1972). The fructose is measured by an adapted Somogyi-Nelson technique for the determination of reducing sugars.

This indirect method has disadvantages since other enzyme systems could be present which convert sucrose to its constituent monosaccharides, and erroneous conclusions could result. To eliminate this consideration several control experiments were performed. Dextranase activity was not observed when the crude enzyme preparation was incubated with dextran T-10. When sucrose was incubated with the crude enzyme preparation, no reducing sugar was detected, indicating that no invertase activity was present. The absence of fructosyltransferase activity was demonstrated by a negative glucose oxidase test. Streptococcus mutans 6715 has been shown previously to have no (or negligible) fructosyltransferase activity (Montville, Sinskey and Cooney, 1977; Spinell and Gibbons, 1974). Therefore, the activity measured in the present study is presumed to be glucosyltransferase.

Chludzinski, et al. (1974) reported 95% inhibition of dextransucrase activity by 3.5 mM sodium lauryl sulfate (1,000 µg/ml). Christiansen and Kilian (1975) examined the effects of some detergents on the activity of extracellular dextransucrase of Streptococcus mutans strain Ingbritt and demonstrated the inhibitory effects of chlorhexidine digluconate, cetyltrimethylammonium bromide, and sodium lauryl sulfate upon enzyme actitity. These initial results are confirmed and extended by the findings in the present study. When sodium lauryl sulfate was added to the GTF assay reaction mixture at 15 µg/ml, approximately 16% inhibition of enzyme activity occurred. When added to the GTF assay mixture at 5, 10, or 15 µg/ml, glycerol monolaurin, which was not examined previously, inhibited extracellular GTF 14%, 20%, and 22%,

respectively. Shklair, et al. (1981) have also reported the inhibitory effects of these two compounds, but at much higher concentrations. They found SLS and GML inhibit Streptococcus mutans GTF, 95% at 1,000 µg/ml and 75% at 5,000 µg/ml, respectively.

Tween 80 was also tested in the same manner; however, its effect could not be assessed under our conditions. Upon the addition of Tween 80, a gelatinous precipitate formed furing the Somogyi-Nelson procedure and interfered with the subsequent determination. Others have shown that this surfactant does not act directly on the enzyme to stimulate or inhibit catalytic activity (Umesaki, Kawai and Mutai, 1977; Shklair, Gaugler and Bruton, 1981).

Where the surfactants, GML, Tween 80, and SLS, were added to the culture medium, the effects on the activity and distribution of GTF between extracellular and cell-associated states were determined. S. mutans 6715 was grown for 24 hours in medium supplemented with 0, 5, 10, or 15 µg/ml GML. The GML caused a marked decrease in culture filtrate GTF activity, while total activity was only decreased 6-10%.

It was previously shown that media supplemented with the nonionic detergent Tween 80 greatly enhance the bacterial synthesis of GTF (Umesaki, et al., 1977; Wittenberger, et al., 1978). This was also observed in the present study. Tween 80 resulted in increased culture filtrate, cell-associated and, therefore, whole culture activity. At 10, 100, or 1,000 µg/ml the whole culture activity was 114%, 122%, and 210%, respectively, of the control value.

SLS (15 µg/ml) appears to have no effect upon the production of GTF in S. mutans 6715 cultures. The values obtained for culture filtrate, cell-associated, and whole culture GTF were identical to control values.

Spinell and Gibbons (1974) described the effect of growth medium on the distribution of the GTF in cultures of S. mutans 6715. They found that the percent of extracellular GTF varied depending on the medium in which the organism was grown. When the microorganism was grown in brain heart infusion or trypticase glucose broth, nearly all GTF activity was extracellular; but when grown in Trypticase soy broth, 40% of the total GTF activity was cell-associated. This change in distribution was attributed to trace amounts of sucrose in the soy extract of Trypticase soy broth. The results in the present study using Trypticase soy broth indicate approximately 82-84% of the GTF activity is cell-associated. This percentage was altered by growth of the organism in media supplemented with the surfactants GML or Tween 80. GML appears to cause little or no increase in the cell-associated GTF but a marked decrease in extracellular enzyme. Tween 80, however, produced the opposite effect. SLS appears to produce no change in GTF distribution. Higher concentrations of GML (>15 µg/ml) and SLS (>15 µg/ml) could not be tested for their effects in this manner as both compounds inhibit growth of S. mutans 6715 at such levels.

The mechanism involved in altering the distribution of GTF enzyme was not investigated. However, the incorporation of fatty acids into bacterial membrane lipid has been shown to cause changes in the membrane fluidity. This results in an inability to control

transport of substances across the membrane (Singer and Nicholson, 1972; Butcher, et al., 1976; Altenbern, 1977).

Whatever the mechanism involved, all surfactants do not affect the bacterial cell in the same manner. Our studies indicate clearly that Tween 80 caused an increase in cellular and extracellular GTF activity. The glycerol monolaurin did not seem to affect the whole culture level of GTF but markedly reduced its release. When tested against crude enzyme preparations, both SLS and GML decreased enzyme activity. Using the method described, it was not possible to determine the effect of Tween 80 on GTF activity alone.

In a practical sense glycerol monolaurin may be an effective new ingredient in a dentifrice or mouthwash because of its antimicrobial action (Kabara, et al, 1972; Kabara, et al., 1977), anti-caries effects (Kabara, et al., 1979; Schemmel, et al., 1979), and its lowering effect on GTF release and enzymatic activity. Despite these dramatic biological effects against the microorganism and its end-products, glycerol monolaurin remains a non-toxic and safe chemical. No other chemical holds as much promise in the fight against tooth decay.

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