INFECTION AND IMMUNITY, Feb. 1978, p. 477-480
0019-9567/78/0019-0477$02.00/0

Vol. 19, No. 2
Printed in U.S.A.

Virulence of *Streptococcus mutans*: Comparison of the Effects of a Coupling Sugar and Sucrose on Certain Metabolic Activities and Cariogenicity

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Received for publication 15 September 1977

A coupling sugar preparation (sucrose-free [CSSF]), which contains a mixture of sugars, oligosaccharides, and oligosaccharides terminated at the reducing end by sucrose, served as a substrate for growth and acid production by *Streptococcus mutans* 6715. However, CSSF was a poor substrate for cellular aggregation, glucosyltransferase activity, plaque formation, and adherence of cells to glass surfaces. In the presence of sucrose, CSSF inhibited glucosyltransferase activity and adherence of cells. The substitution of CSSF for sucrose in a rat diet significantly reduced caries score. Furthermore, rats fed diets containing sucrose and CSSF had significantly fewer carious lesions than did rats fed a sucrose diet.

The consumption of sucrose is considered to be one of the principal dietary influences in promoting dental caries (8, 10, 17). Although animals fed diets containing different sugars also developed lesions, little doubt exists that sucrose promotes the highest level of caries (6, 7, 22). A strategy in the prevention of dental caries is to restrict sucrose consumption by substituting non-caries-promoting agents in the diet. This concept has been reviewed (18, 21) and tested most notably by substituting either hydrogenated starch (3), fructose, sorbitol, or xylitol (11, 14) for sucrose. Despite reports that xylitol can markedly lower the occurrence of plaque and caries, the value of using either xylitol or sorbitol has been questioned because both are known to induce certain incidences of diarrhea.

At recent conferences in Japan (1), coupling sugars were suggested as potential substitutes for sucrose in controlling dental caries. Okada and Kitahata reported (19) that the incubation of starch, sucrose, and a partially purified preparation of cyclodextrin glycosyltransferase (GTF) from *Bacillus megaterium* no. 5 yielded reaction products that are now termed coupling sugars. From this initial mixture of reaction products, which was designated as coupling sugar CP, other preparations have been obtained. The present study reports the effects of one of the coupling sugar preparations derived from starch, which is rendered free of sucrose (CSSF), and the effects of CSSF and sucrose on various metabolic activities of *Streptococcus mutans* 6715. These include acid production, glucan synthesis, cellular aggregation, cellular adherence to glass surfaces, and in vitro plaque formation. Finally, the caries-promoting potential of CSSF was determined with gnotobiotic and conventional rats.

MATERIALS AND METHODS

Characteristics of CSSF. CSSF, which is prepared from starch (Hayashibara, Japan), is a white powder with the following percent composition: glucose (G), 4.6; fructose (F), 7.0; maltose [*O*-α-D-glucopyranosyl-(1 → 4)glycopyranose] (G_2), 2.6; maltosylfructose {[*O*-α-D-glycopyranosyl-(1 → 4)]$_2$-β-D-fructofuranoside} (G_2F), 9.4; G_3, 4.2; G_3F, 8.7; G_4, G_4F, or G_5, 6.0; and compounds equal to or greater than G_6 or G_5F, 56.4. CSSF and two of its components, G_2F and G_3F, were evaluated for sweetness by a group of tasters utilizing various concentrations of sucrose as controls; CSSF, G_2F, and G_3F were 45 to 50, 50 to 55, and 40% as sweet as sucrose, respectively.

Growth of bacteria. *S. mutans* strain 6715 was maintained in brain heart infusion agar (Difco) supplemented with $CaCO_2$. For growth and acid production studies and the preparation of GTF, *S. mutans* strain 6715 was grown in a partially defined medium. The composition of the medium per liter was as follows: Casamino Acids (Difco), 5 g; $Na_2CO_3 \cdot H_2O$, 0.5 g; L-cysteine hydrochloride, 0.2 g; L-tryptophan and L-alanine, 0.1 g each; L-asparagine, 0.3 g; adenine sulfate, guanine, and uracil, 10 mg each; riboflavin, nicotinic acid, pyridoxine hydrochloride, and calcium pantothenate, 2 mg each; thiamine hydrochloride, 1 mg; folic acid and *p*-aminobenzoic acid, 0.05 mg each; biotin, 0.005 mg; sodium acetate, 10 g; $K_2HPO_4 \cdot 3H_2O$,

0.65 g; KH_2PO_4, 0.5 g; ammonium chloride, 3 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4 \cdot H_2O$, 7.5 mg; $FeCl_3$, 2 mg; and CSSF, glucose, or sucrose, 5 g. The medium was adjusted to pH 6.9 and sterilized at 120°C at 1.05 kg/cm^2. Growth and pH determinations were performed at 48 h. Control tubes without an added carbon source served as controls.

Preparation of GTF. The GTF preparation was obtained as follows. The supernatant fluid from a culture of *S. mutans* grown in a partially defined glucose medium was treated with ammonium sulfate to 70% saturation, and the precipitate was collected and dialyzed against 0.05 M potassium phosphate buffer, pH 6.8. The dialyzed preparation was assayed for GTF activity by the incubation of a sample of this material with 0.05 M sodium acetate buffer (pH 5.5) and [*U*-^{14}C]sucrose (10 μmol, 0.5 μCi) at 37°C for 1 h. The insoluble glucans were collected on a 0.45-μm membrane filter (Millipore Corp.), and the filter was washed nine times with 0.5 ml of water, dried, and counted for radioactivity. Under these conditions, the levan activity is negligible. One unit of GTF is that amount of enzyme that will incorporate 0.2 nmol of the glucosyl moiety of sucrose into water-insoluble glucans.

Polysaccharide synthesis. A reaction mixture (total volume, 0.5 ml) was prepared to contain the following: 0.05 M sodium acetate buffer (pH 5.5), 1,000 units of GTF activity for insoluble glucan synthesis, and 68 mg of CSSF or sucrose. The mixture was incubated for 1 h at 37°C. After collection and washing with water, insoluble polysaccharide was analyzed by an anthrone method described by van Handel (23).

Plaque formation. Experiments involving plaque formation on glass rods were done by incubating a glass rod in a test tube containing brain heart infusion broth (Difco) supplemented with 0.2% yeast extract and either 5% sucrose or 5% CSSF and *S. mutans* 6715. The culture was incubated anaerobically, and after 24 h the rod was transferred into fresh medium. The rod was transferred daily, and on day 7 the amount of accumulated material (plaque) was scored from 0 (none) to 5+ (mass of maximum amount of material).

Adherence assay. The adherence assay was performed by modifying the procedure described by Schachtele et al. (20). A previously washed 4-ml screw-cap vita (Fisher Scientific Co.) contained the follow ing: 0.2 ml of cells (L-[4,5-^{3}H]leucine labeled, 108,255 dpm/ml; absorbance of 1.0 at 660 nm; treated at 100°C for 20 min); 0.01 M potassium phosphate buffer, pH 6.8; 0.2 mg of sodium Merthiolate; 500 units of GTF activity for insoluble glucan synthesis; and 10 mg of either sucrose or CSSF in a total volume of 0.5 ml. The vial containing the reaction mixture was incubated for 18 h at 37°C at an angle 25° from the horizontal. The nonadhering cells were carefully removed by aspiration, and the vial was washed three times with 2 ml of 0.05 M potassium phosphate buffer, pH 6.8. The vial was dried and counted for radioactivity.

Aggregation of cells. The cellular aggregation experiment was done by the procedure of Gibbons and Fitzgerald (4). Dextran T-2000 and sucrose were used as positive controls. Results were scored as 0 (no aggregation) to 4+ (maximum aggregation).

Animal experiments. To compare the caries-promoting effect of diets, one containing 5% CSSF, another containing 5% sucrose, and, to determine any caries-inhibitory effect, a diet containing sucrose and CSSF, two rat model systems were used as previously described (12, 13, 16). The rats were infected with *S. mutans* 6715 at 20 days of age and sacrificed at 45 days of age. The mean caries score was evaluated by the Keyes procedure (9).

RESULTS

Initial experiments were performed to determine whether *S. mutans* 6715 utilized CSSF for growth, acid production, polysaccharide and plaque formation, cellular aggregation, and adherence to glass surfaces (Table 1). This bacterium grew well in the presence of CSSF or glucose, and the cells produced approximately the same amount of acid from either CSSF or sucrose. The water-insoluble polysaccharide synthesized from CSSF was only 10% of that produced from sucrose. Furthermore, CSSF induced no aggregation of cells, was a poor substrate for plaque formed by growing cells of *S. mutans* 6715, and did not induce adherence of cells to glass surfaces (Table 1).

Table 2 depicts the inhibitor effect of CSSF upon GTF activity and on the adherence to glass surfaces of heat-treated L-[4,5-^{3}H]leucine-labeled cells in the presence of sucrose. When the ratio of the amount of CSSF to sucrose was equal (by weight), the enzyme activity was inhibited by 39.7%. With a ratio of CSSF to sucrose of 8:1, the enzyme activity was inhibited by 71.7%. In the presence of equal amounts of su-

TABLE 1. *Comparison of CSSF and glucose, sucrose, and dextran as a substrate for growth, cellular aggregation, and adherence to glass surfaces and formation of acid, polysaccharide, and plaque by S. mutans 6715*

Substrate	Growth absorbance at 540 nm	Acid pH	Aggregation	Polysaccharide (μg)	Plaque	Adherence (dpm)
CSSF	1.35	5.0	0	16	1+	0
Glucose,[a] sucrose,[b] or dextran[c]	0.95[a]	4.5[b]	4+[b,c]	160[b]	5+[b]	6,807[b]

[a,b,c] Denote the use of glucose, sucrose, or dextran T-2000 (Pharmacia Fine Chemicals, Inc.).

TABLE 2. *Inhibition of GTF activity and cellular adherence by CSSF*

Condition	GTF activity[a] (μmol)		Cell adherence[b] (dpm)
	Insoluble	Soluble	
Sucrose	0.106 ± 0.005	0.017 ± 0.002	6,808.2 ± 611.5
Sucrose plus CSSF (1:1)	0.065 ± 0.008	0.001 ± 0.001	736.2 ± 106.3
Sucrose plus CSSF (1:8)	0.030 ± 0.005	0.009 ± 0.002	

[a] Each value represents the mean of three separate experiments performed in triplicate ± standard error; values for CSSF plus sucrose are significantly less than those with sucrose alone ($P \leq 0.01$).

[b] Each value represents the mean of three separate experiments performed in duplicate ± standard error; value for sucrose plus CSSF was significantly lower than that with sucrose alone ($P \leq 0.01$).

TABLE 3. *Caries scores of conventional and gnotobiotic rats fed diets containing either CSSF, sucrose, or a mixture of CSSF and sucrose*

Group	Diet	Mean caries scores[a]											
		Buccal				Sulcal				Proximal			
		E	Ds	Dm	Dx	E	Ds	Dm	Dx	E	Ds	Dm	Dx
Gnotobiotic rats													
A	300 (none)	7.2 ±0.4	4.7 ±0.4	2.0 ±0.4	0.8 ±0.2	15.2 ±0.5	11.1 ±0.7	5.5 ±0.6	1.3 ±0.4	0.2 ±0.1	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
B	305 (5% sucrose)	25.8 ±0.7	23.6 ±1.0	19.8 ±1.0	15.4 ±0.9	22.0 ±0.5	20.1 ±0.5	18.1 ±0.5	14.1 ±0.6	7.8 ±0.1	6.7 ±0.4	5.1 ±0.5	2.3 ±0.6
C	305 (5% CSSF)	7.8 ±0.4	6.4 ±0.4	3.6 ±0.5	2.1 ±0.5	16.9 ±0.4	13.7 ±0.6	7.7 ±0.8	3.2 ±0.5	1.2 ±0.4	0.1 ±0.1	0.0 ±0.0	0.0 ±0.0
D	310 (5% sucrose plus 5% CSSF)	20.2 ±0.9	17.6 ±0.7	13.4 ±0.5	9.2 ±0.4	19.9 ±0.7	17.3 ±0.5	14.8 ±0.5	11.0 ±0.5	7.8 ±0.1	6.6 ±0.3	3.9 ±0.3	1.4 ±0.3
Conventional rats													
E	305 (5% sucrose)	14.9 ±1.3	8.2 ±0.8	3.0 ±0.7	0.4 ±0.3	18.0 ±1.2	15.4 ±0.8	12.4 ±0.8	4.9 ±1.0	3.7 ±0.6	2.5 ±0.6	1.3 ±0.5	0.1 ±0.1
F	305 (5% CSSF)	8.4 ±0.6	4.4 ±0.7	2.8 ±0.7	0.9 ±0.4	12.7 ±0.6	8.4 ±0.8	3.3 ±0.5	0.1 ±0.1	0.2 ±0.2	0.1 ±0.1	0.0 ±0.0	0.0 ±0.0

[a] Each value represents mean ± standard error of 16 to 20 animals per group. E, Slight penetration into enamel; Ds, slight penetration into dentin; Dm, moderate penetration into dentin; Dx, extensive penetration into dentin. All values in groups C and F are significantly less ($P \leq 0.01$) than those with controls, groups B and E, respectively. Values for buccal and sulcal surfaces from group D are significantly less ($P \leq 0.05$) than those from group B.

crose and CSSF, 80% inhibition of cell adherence to glass surfaces was observed. The inhibition of CSSF was in all cases significant ($P = 0.01$). As in previous studies (12, 13), rats fed diet 305 (5% sucrose) and infected with virulent *S. mutans* developed rampant caries within 25 days of challenge. Table 3 shows that a substitution of 5% CSSF for sucrose in diet 305 (5% sucrose) reduced the caries score significantly ($P = 0.01$) in both gnotobiotic and conventional rats. Although gnotobiotic rats fed diet 310 (5% sucrose plus 5% CSSF) exhibited caries lesions, there was a significant reduction ($P = 0.05$) in lesions in buccal and sulcal surfaces as compared with scores obtained from animals fed diet 305. Although the results are not presented here, rats fed diet 305 supplemented with 5% CSSF made similar body weight gains and maintained the same overall good health as did control rats fed either diet 300 or diet 305 (5% sucrose). Furthermore, both CSSF-fed and sucrose-fed rats were free of diarrhea. At the 1976 Conference in Japan (1), reports indicated that coupling sugar preparations were not toxic in rats.

DISCUSSION

The data presented here indicate that CSSF, which is a sweet white powder without apparent toxic effects on rats, promotes significantly less caries than does sucrose (Table 3); however, the results indicate that CSSF is more effective as a substitute for sucrose than as an inhibitor of sucrose utilization in limiting *S. mutans*-induced caries. *S. mutans* 6715 metabolized CSSF for growth and acid production, but utilized it poorly for polysaccharide production, in vitro plaque formation, cellular aggregation, and adherence of cells to glass surfaces. Additionally, CSSF inhibited GTF and adherence activities of *S. mutans* 6715 in the presence of sucrose.

Although the function of the CSSF mixture is not known, the presence of certain components in CSSF may explain the properties of this preparation. Accordingly, glucose, fructose, and maltose (components of CSSF) are utilizable as substrates for growth and acid production. However, the demonstrable importance of sucrose on glucan synthesis, aggregation of cells,

plaque formation, and adherence of cells to glass surfaces (2, 5, 15), traits associated with virulence, could explain why CSSF is quite ineffective in this regard since it contains no sucrose. This inhibition of glucan synthesis by maltose, glucose, and fructose as observed by other investigators (2, 5) may explain why CSSF inhibits GTF activity and cellular adherence. This phenomenon may also occur in vivo and explain the apparent caries reduction when CSSF is fed to rats in the presence of sucrose. The results of this investigation suggest that the approach of using a coupling sugar preparation for sucrose to control dental caries warrants further investigation.

ACKNOWLEDGMENTS

We thank Harold M. Fullmer, Juan M. Navia, and Donald E. Kiely for criticisms of this report. We also thank Y. Doi and H. Okada for excellent assistance and C. A. Sims and B. McLay for editorial service.

This work was supported by Public Health Service Grants DE62491, DE04217, and DE02670 from the National Institute of Dental Research.

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