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In Vitro Colonization of *Streptococcus mutans* on Enamel

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An in vitro model consisting of enamel from extracted human molars, suspended from wires in inoculated culture tubes, was used to study the adhesion of bacteria to enamel. Under conditions in which there was no macroscopically visible plaque formation, electron micrographs showed no bacterial deposits on the enamel surface. In samples where *Streptococcus mutans* attached to enamel, an extracellular, pellicle-like material was associated with the bacteria adjacent to the enamel. This material appeared to bind to the enamel surface and to mediate bacterial attachment. Membrane-filtered (Millipore Corp.) saliva deposited a thin surface layer on the enamel, but there were no observable alterations of *S. mutans* attachment to enamel pretreated with saliva. It was noted that Bratthall serotype *c* and *e* strains of *S. mutans*, when grown in glucose-containing medium, attached, although less tenaciously, to enamel and nichrome wires. Chemical and gas chromatographic analyses of cell-associated materials formed by serotype *c* and *e* strains cultured in glucose-containing medium revealed low amounts of glucose-positive material and no polymer linkages characteristic of glucan; yet the same strains cultured in sucrose-containing medium had relatively high amounts of glucose-positive material, with polymer linkages characteristic of glucan. Serotype *a*, *b*, and *d* strains could attach only in sucrose-containing media.

Bacterial plaque formation on tooth enamel may be dependent on two types of adhesion phenomena. Organisms must sorb to the tooth or to organic films on the enamel surface, and organisms must be able to adhere to each other as the plaque enlarges. The abilities of certain bacteria to become attached to enamel and to proliferate while localized there seem to be the major ecological determinants influencing formation of plaque and pathogenicity (11, 41). The events and mechanisms are, however, not completely understood.

A large body of evidence strongly implicates *Streptococcus mutans* as a prime pathogen in dental caries of humans and experimental animals (2, 5, 7, 10, 22, 24). *S. mutans* is known to form glucans from sucrose via glucosyl transferase activity, and the synthesis of cell surface-associated glucans from sucrose has been thought essential for this species to form bacterial accumulations termed dental plaque (9, 11, 13, 21, 25, 29, 30, 35, 40, 41, 50).

Most models for bacterial attachment to tooth surfaces involve growth of bacteria on wires, glass, plastic, or hydroxyapatite powder. Few ultrastructural studies of plaque formation have used intact tooth enamel (46). However, because of the purported structural distinctness of enamel, its characteristic wettability (14), and the possible specificity of bacterial-enamel interactions (12), the early attachment of bacteria to tooth enamel in vitro rather than to enamel substitutes may better parallel events in vivo. The recent development of a technique for making thin sections of the enamel-plaque interface (45) has enabled a study of enamel-bacteria interactions. The purpose of this investigation, therefore, is to describe some of the parameters involved in the early plaque formation by *S. mutans* on enamel and nichrome wire in vitro.

MATERIALS AND METHODS

Enamel preparation. Enamel specimens, approximately 40 mm^2, were cut tangentially to the surface of extracted, tap water-stored human molars with a carborundum disk. A hole to accept a 20-gauge nichrome wire was made through the specimens with a dental bur. The specimens were then polished with a slurry of fine pumice to remove surface material and thoroughly washed in deionized water with an ultrasonic cleaner. Specimens were suspended by wire, placed in stoppered test tubes, and autoclaved. Care was taken to assure that they were not desiccated at any stage.

Microorganisms, media, and growth. *S. mutans* strains NCTC 10449 (Bratthall serotype *c*) and 6715-13 (Bratthall serotype *d*), both known to be good plaque formers in vivo and in vitro and to be cariogenic (9, 30, 41, 42), and *S. faecalis* ATCC 9790, which fails to form plaque, were primarily studied. Additionally, plaque formation was studied macroscopically and chemically with the following Bratthall serotype-rep-

resentative strains of *S. mutans*: E-49 and OMZ-61 (serotype *a*); FA-1 and BHT (serotype *b*); GS-5 and Ingbritt-1600 (serotype *c*); OMZ-176 and SL-1 (serotype *d*); and LM-7 and B-2 (serotype *e*).

Stock cultures were maintained by monthly transfer in fluid thioglycolate medium (Difco) supplemented with meat extract (20%, vol/vol) and excess $CaCO_3$. For experiments, cultures were adapted to growth in the following media: the complex medium of Jordan et al. (20), supplemented with 50 mg of Na_2CO_3 per liter and either 5% sucrose or 5% glucose; or FMC, a defined medium of Terleckyj et al. (44), containing either 5% sucrose or 5% glucose.

For plaque growth, media were inoculated with 0.2 ml of a 24-h culture of the various strains adapted to the same culture medium, and the sterile wires or wire-mounted enamel specimens were suspended in the stoppered culture tubes for growth at 37°C (43). These plaque supports were transferred daily to uninoculated broth. At various times, enamel specimens were fixed and further processed for electron microscopy as detailed below, or scored for plaque on wires and enamel by the method of McCabe et al. (26), or chemically analyzed as detailed below.

To observe any ultrastructural effects of sterile saliva on enamel and on colonization by bacteria, enamel specimens were incubated for 1 h at 37°C in 5 ml of filter-sterilized (0.45-μm pore diameter, type HAWP; Millipore Corp., Bedford, Mass.), stimulated, pooled human saliva.

Electron microscopic preparation. The specimens processed for electron microscopy were removed from the growth medium, fixed with 2.5% glutaraldehyde in 390 mosM phosphate buffer (pH 7.4) and postfixed in 1% osmium tetroxide in Veronal buffer (pH 7.3) (53). They were then washed in the phosphate buffer and placed in an acidic gel containing 0.1 N HCl–15% gelatin (Baltimore Biological Laboratory, Cockeysville, Md.) for 3.5 h to demineralize slightly the enamel surface (46). After dehydration in acetone and embedment in Spurr's epoxy medium (39), the resin was polymerized at 70°C. Sections 500 μm thick were cut perpendicularly to the enamel surface with a diamond wheel, using a Bronwill sectioning machine.

The sectioned specimens were re-embedded, and, after polymerization, the enamel was ground from the inner surface with a sandpaper wheel followed by hand grinding with 600-grit sandpaper (Beuhler Ltd., Evanston, Ill.) until only 1 to 5 μm of outer surface enamel remained. (A 1- to 5-μm thickness of enamel could be estimated with a dissecting microscope because the enamel was completely transparent, yet still visible in oblique light.) The specimens were re-embedded for a third time so that the plastic completely surrounded the enamel remnant. Thin sections were prepared with a Reichert ultramicrotome, using a diamond knife. Silver-gold-colored sections were stained with aqueous uranyl acetate followed by lead citrate (52) and examined at 90 kV with a Zeiss EM10 electron microscope.

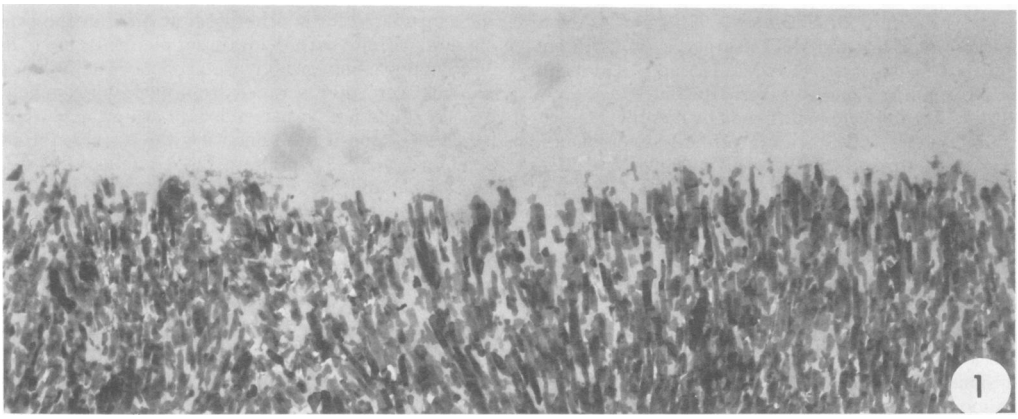
Analysis of glucans and DNA. Plaques of various strains were grown by serial daily passage of duplicate wires to tubes of uninoculated defined medium containing either 5% sucrose or 5% glucose. After 5 days, plaque scores were recorded, and the in vitro plaques were incubated for another 24 h at 37°C in the same medium without carbohydrate supplementation to deplete endogenous carbohydrate pools. The adherent deposits were then scraped from each wire, pelleted by centrifugation, resuspended in water, and repelleted. The deposit from one wire was extracted in hot perchloric acid (33) prior to DNA analysis, whereas that of a duplicate wire was boiled for 2 h in 4 N H_2SO_4 (16) prior to assay of glucose oxidase-positive material. After neutralization with KOH, DNA was analyzed by the method of Burton (3), using either calf thymus DNA or 2-deoxy-D-ribose as a standard. Glucose was quantitated by the glucose oxidase procedure (Glucostat, Worthington Biochemicals Corp., Freehold, N.J.) in 500 mM tris(hydroxymethyl)-aminomethane buffer, pH 7.0.

Analysis of glucan linkages. The deposits adherent to the bottom and sides of a 500-ml Erlenmeyer flask, which had been used to contain a 200-ml 5% glucose- or 5% sucrose-supplemented defined-medium culture of *S. mutans* NCTC 10449, were washed extensively with distilled water to remove residual medium and nonadherent cells. The deposit was scraped from the surface, washed with distilled water by centrifugation (10,000 × *g*, 10 min, 0°C), and resuspended in a known volume of distilled water from which a sample for DNA analysis (3) was removed. The cell-associated glucan fraction was extracted with 1 N NaOH (37°C, 60 min). This material, designated AS, was further treated with 4.3 N KOH at 100°C for 1.5 h, neutralized with HCl, and dialyzed against running distilled water for 20 h. Any precipitate forming in the dialysis bag was removed by centrifugation, as described, and designated AS-I. No precipitate formed in the supernatant fluid when an equal volume of 100% ethanol was added and the sample was chilled.

Samples of the putative glucans were analyzed for glucose by the glucose oxidase method as described above. Hydrolyzed commercial dextran served as the standard. Additional samples were methylated for glucan linkage analysis by gas chromatography (1, 8). A Hewlett-Packard model 5710 A gas chromatograph (flame ionization detector) was used with a stainless-steel column (190 by 0.15 cm) containing 3% OV-225 on Gas Chrom Q (Applied Sciences). Nitrogen was the carrier gas (20 ml/min), and the column temperature was 170°C. The four chromatogram peaks of partially methylated alditol acetates corresponded to chain ends, 1 → 3, 1 → 6 linkages, and branch points and were identified in lieu of mass spectrometry by the similarity of their retention times, *T* values = 1.0, 1.82, 2.22 and 4.21, respectively, to published values (23).

RESULTS

Enamel specimens which were incubated for 2 days in sterile complex culture medium at either pH 7.2 or 5.0 showed no film on their surfaces (Fig. 1). However, specimens incubated for only 1 h in filter-sterilized saliva exhibited a uniformly wide (0.01 μm) film covering the enamel surface (Fig. 2). Enamel incubated in complex medium with a non-plaque-forming microorganism, *S. faecalis*, showed neither adherent



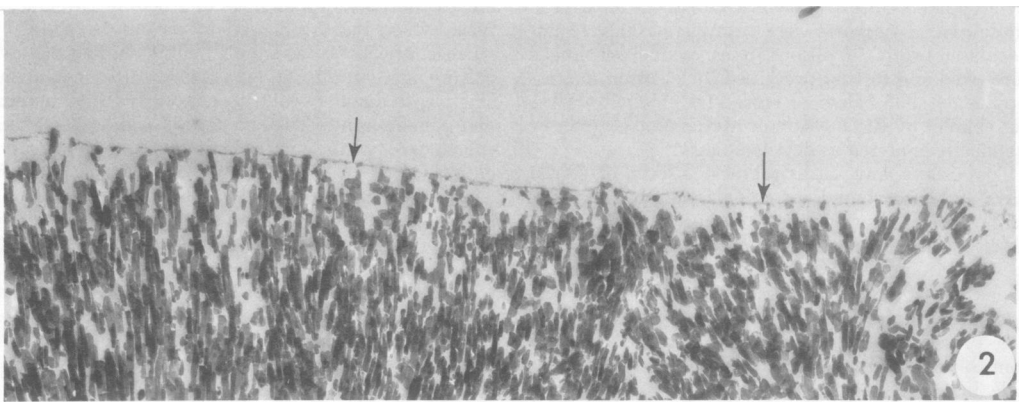


FIG. 1. *Transmission electron micrograph of polished surface enamel after incubation for 2 days in complex medium containing 5% sucrose (pH 5). No visible organic material is on the surface. ×21,000.*

FIG. 2. *Electron micrograph of polished enamel which has been incubated in Millipore-filtered (0.45 μm) saliva for 2 h. A thin (0.01 μm) pellicle (arrows) covers the surface crystallites. ×19,000.*

microorganisms nor films. In addition, *S. faecalis* did not attach to saliva-coated enamel.

S. mutans NCTC 10449 incubated in complex medium (Fig. 3) or in chemically defined medium, both containing 5% sucrose (Fig. 4), attached to the surface of the enamel. The attachment was associated with the presence of a thin (0.01 to 0.1 μm) electron-dense film. There were no apparent ultrastructural differences in the attachment of this plaque-forming organism in protein-containing complex medium or in protein-free medium. Also, no visible alterations in strain 10449 attachment to enamel pretreated with filter-sterilized saliva were noted, and it was not possible to determine whether the surface films were exclusively of bacterial or salivary origin (Fig. 5).

Ultrastructurally, the attachment of *S. mutans* 10449 (serotype *c*) to enamel was similar in both sucrose-containing and glucose-containing media (Fig. 6A and B). Although strain 6715-13 (serotype *d*) attachment to enamel in the presence of sucrose was similar to that of strain 10449 (Fig. 7A), there was neither bacterial attachment nor observable film production on the enamel incubated with *S. mutans* 6715-13 in glucose-containing medium (Fig. 7B).

Adherent deposits of strain NCTC 10449 cultured in sucrose-containing medium yielded cell-associated, glucose-rich, alkali-solubilized material which was shown by gas-liquid chromatography to contain significant amounts of $1 \rightarrow 3$ and $1 \rightarrow 6$ linkages as well as branching residues with linkages to carbon atoms 1, 3, and 6 (Table 1). More alkali-solubilized material per microgram of DNA was produced by strain NCTC 10449 cultured in glucose- than in sucrose-supplemented medium, but neither was this material glucose rich nor were any gas-liquid chromatographic peaks consistent with dextran- or "mutan"-like polymers detected. Chemical analysis of strain LM-7 as well as of strain NCTC 10449 revealed relatively low levels of glucose-positive material in the adherent deposits when

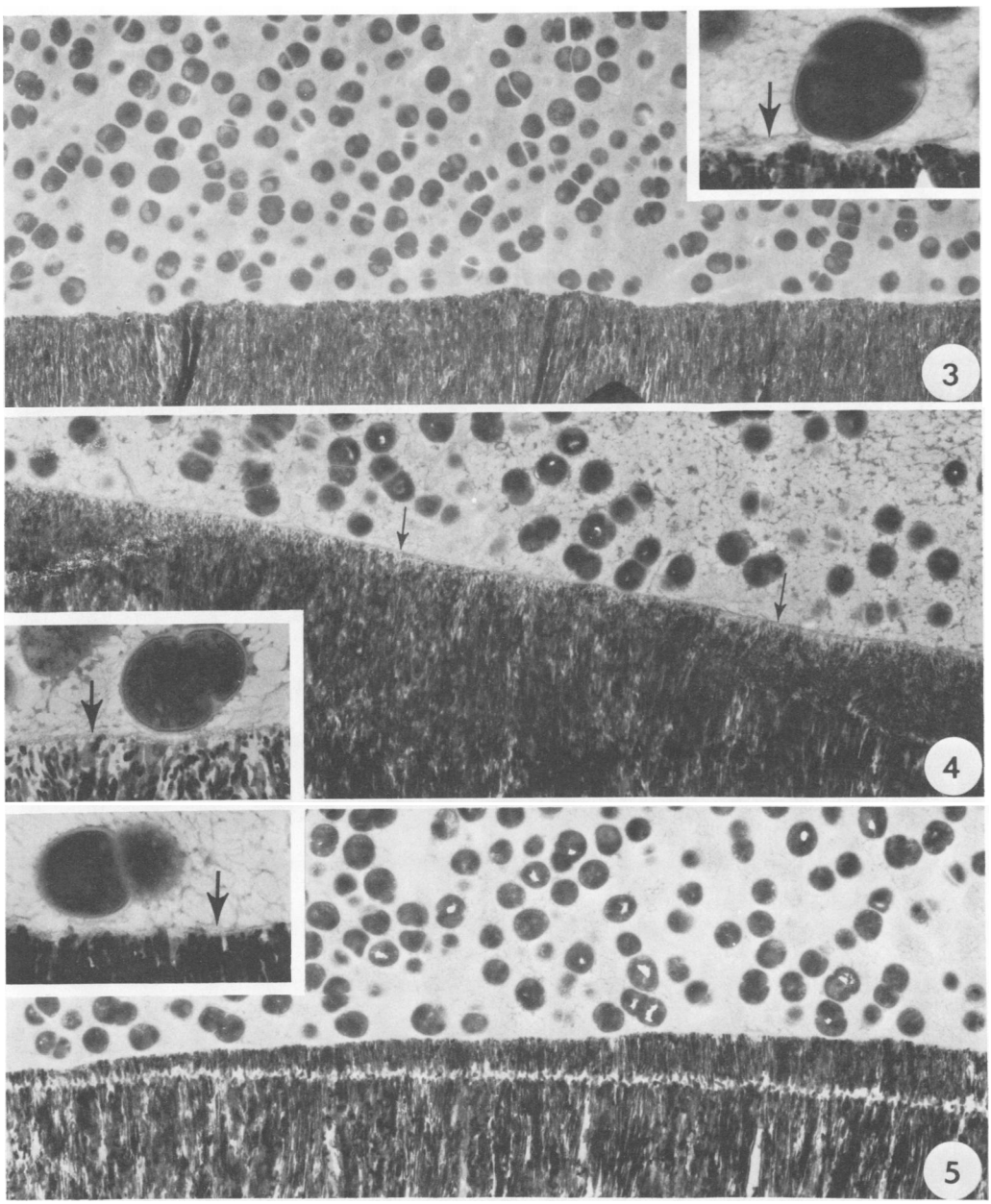


FIG. 3. *Electron micrograph of enamel-bacteria interface after incubation of S. mutans NCTC 10449 with enamel for 2 days in chemically defined medium containing 5% sucrose. Bacterial association with the enamel is evident at low magnification (×7,600). The inset shows apparent bacterially derived extracellular material (arrow) associated with the attachment of the microorganisms to the enamel (×34,000).*

FIG. 4. *Electron micrograph of enamel-bacteria interface after incubation of S. mutans NCTC 10449 with enamel for 2 days in complex medium containing 5% sucrose. Bacterial association with the enamel is evident at low magnification (×8,500). The inset shows an apparent bacterially derived extracellular material (arrow) associated with the attachment of the microorganisms to the enamel (×27,000).*

FIG. 5. *Electron micrograph of the enamel-bacteria (S. mutans NCTC 10449) interface. Enamel was incubated for 1 h in Millipore (0.45 μm)-filtered saliva before placing it in inoculated complex broth. No difference is observed in bacterial attachment or surface films from specimens that had not been exposed to saliva (low magnification, ×6,900; high magnification, ×34,000).*

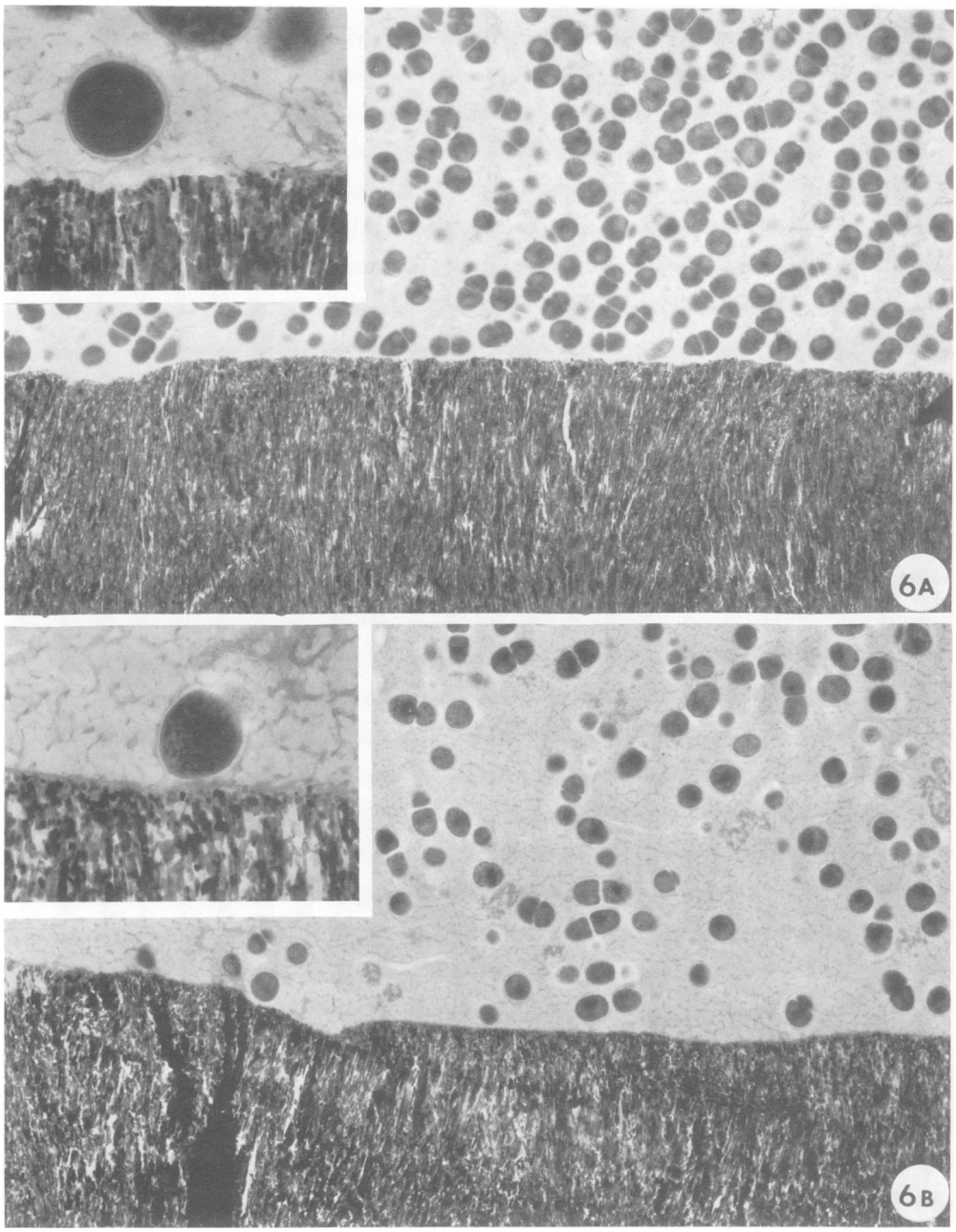


FIG. 6. *Sections from enamel incubated with S. mutans NCTC 10449 in either chemically defined medium containing 5% sucrose (A) or chemically defined medium containing 5% glucose (B). (Low magnification, ×7,500; high magnification, ×22,000).*

cultured in glucose-supplemented medium as opposed to those cultured in sucrose-supplemented medium (Tables 1 and 2).

Macroscopically, plaque formation on enamel and the suspending wires was noted in all 12 *S. mutans* strains cultured in sucrose-containing media, with plaque formation on enamel consistently correlating with that on wires (Table 2). However, plaque formation by strains of Bratthall serotypes *c* and *e* was also observed in

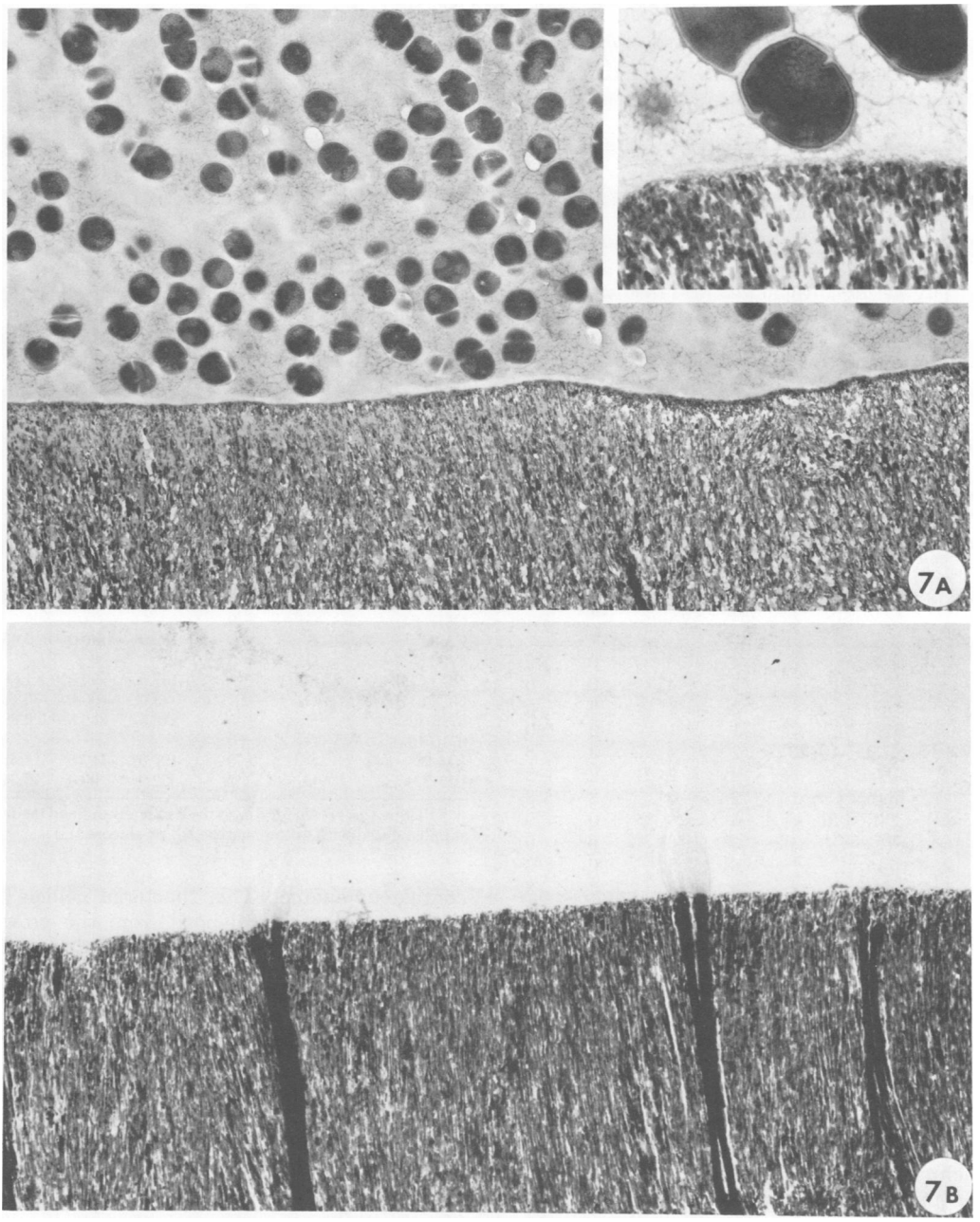


FIG. 7. *Electron micrographs of enamel incubated with S. mutans 6715-13 in chemically defined medium containing either 5% sucrose (A) or 5% glucose (B). Bacterial attachment is evident in the sucrose medium (×10,000; inset, ×22,000) but is not evident in the glucose medium (×8,000).*

glucose-containing media. In the complex medium, adherence in the presence of glucose was similar to that in the presence of sucrose (Table 2), although in defined medium adherence in the presence of glucose for strains of serotypes *c* and *e* was less than in the presence of sucrose (Table 3), as revealed by the relative ease with which the plaques were dislodged.

DISCUSSION

Some of the concepts of bacteria-enamel interactions have been extrapolated from studies

TABLE 1. *Quantity, glucose analysis, and linkage composition of alkali-soluble, high-molecular-weight material isolated from adherent deposits of S. mutans NCTC 10449 cultured in glucose- or sucrose-containing defined medium*

Fraction[a]	Amt (mg) produced[b]	Glucose (%)[c]	μg of glucose/μg of DNA	mol%[d]			
				Ends	1 → 3	1 → 6	Branches
AS-I sucrose-containing culture	20.9	63	12.8	8	51	30	11
AS-I glucose-containing culture	14.7	0.8	0.66	ND[e]	ND	ND	ND

[a] Isolated after extraction of adherent material with 1 N NaOH at 37°C for 1 h.
[b] Dry weight of lyophilized AS-I fractions.
[c] Based on glucose oxidase analysis of buffered, neutralized, hot acid hydrolysates.
[d] Determined as ends: 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, $T = 1.0$; 1 → 3 linkages: 1,3,5,-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, $T = 1.83$; 1 → 6 linkages: 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methyl-D-glucitol, $T = 2.24$; branches: 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol, $T = 4.29$.
[e] ND, None detected.

TABLE 2. *Plaque scores of representatives of Bratthall serotypes in medium containing sucrose or glucose*[a]

Serotype	Strain	Plaque scores			
		Sucrose		Glucose	
		Enamel	Wire	Enamel	Wire
a	E-49	4	3	0	0
	OMZ-61	5	4	0	0
b	FA-1	2	2	0	0
	BHT	1	1	0	0
c	NCTC-10449	2	2	3	3
	GS-5	2	2	2	2
	Ingbritt	2	2	3	3
d	OMZ-176	3	3	0	0
	SL-1	3	3	0	0
	6715	6	5	0	0
e	LM-7	3	3	3	3
	B-2	3	3	2	2

[a] Scores by the McCabe et al. (25) method were recorded after 4 days on enamel and wire surfaces. Plaques were grown in complex medium as described in the text.

TABLE 3. *Plaque scores on wires and ratio of glucose to DNA for representatives of the five Bratthall serotypes of S. mutans*[a]

Strain	Carbohydrate	Plaque score	Glucose (μg/wire)	DNA (μg/wire)	Glucose/DNA
OMZ-61	S	5	249.5	105.0	2.38
OMZ-61	G	0			
FA-1	S	3	62.5	140.0	0.45
FA-1	G	0			
NCTC-10449	S	5	167.5	90.0	1.86
NCTC-10449	G	1	13.0	80.0	0.16
6715-13	S	5	213.5	242.5	0.88
6715-13	G	0			
LM-7	S	5	190.0	117.5	1.62
LM-7	G	2	22.0	90.0	0.24

[a] Plaque was grown for 5 days in chemically defined medium supplemented with either sucrose (S) or glucose (G).

of marine organisms in which extracellular polymeric material was noted to be necessary for a stable bond between the organisms and hard surfaces (27, 54). Perhaps similar bacterial attachment has recently been observed in dental plaque formed on enamel in vivo (46) in which there was an extracellular material associated with the bacteria adjacent to the enamel surface.

In the present study, pure cultures of *S. mutans* have provided additional information on the events subsequent to bacterial sorption to enamel in vitro. Attachment of plaque-forming microorganisms to enamel appeared to be associated with an extracellular material. This extracellular material was characteristically interposed between the bacteria and the surface of contiguous enamel. This "bacterial pellicle" is not a proteinaceous deposit from the growth medium because: (i) organisms incubated in protein-free medium showed patterns of bacteria-enamel interactions similar to those shown by organisms grown in complex medium; and (ii) specimens incubated in bacteria-free, complex medium (pH 7.2 or 5.0) showed no precipitated organic surface layer. In addition, non-plaque-forming *S. faecalis*, incubated under the same conditions, failed to form enamel surface films or to attach to the enamel. Inasmuch as sucrose was not essential for the development of such films by *S. mutans* serotype *c* or *e* strains, it is not likely to be a glucan.

On enamel in vivo, salivary glycoprotein components are known to rapidly form thin surface coatings termed acquired salivary pellicle (17, 28, 38). Similar films were observed in the present study upon incubation of enamel with saliva for a brief period of time. The possibility that filtered saliva contains polymers of bacterial origin, however, cannot be ruled out and should

be recognized. These films were not requisite for attachment of *S. mutans* to enamel, and no morphological difference could be observed between bacterial attachment to enamel specimens pretreated with saliva and those not pretreated. Others have also demonstrated that glycoprotein-rich (hog gastric) mucin is not essential for attachment of *S. mutans* to nichrome surfaces (26). Salivary films have been shown to enhance adsorption of certain plaque-forming microorganisms to enamel and to inhibit the adsorption of others (4, 18, 34). Thus, salivary coatings may have a role in determining the relative proportion of different bacteria which adsorb to enamel, but they do not appear to be essential for the attachment of *S. mutans*. Furthermore, saliva in vivo is not free from plaque-forming microorganisms, and formation of the acquired enamel pellicle may indeed result from bacterial synthetic processes, as well as from nonbacterial processes.

Plaque formation on enamel correlated closely with that on nichrome wires. The wire model for bacterial attachment (26) may thus be a good predictor of bacteria-enamel interactions. Because the surface characteristics of these two different plaque supports are not likely to be the same, the often stated variables of enamel-bacteria specificity (49), substrate charge (36), and surface energy (14) may not be primary determinants of *S. mutans* attachment.

It is of interest that not all *S. mutans* strains require the presence of sucrose for plaque formation. The sucrose dependency of *S. mutans* to form large deposits has been repeatedly emphasized in the literature (9, 13, 21, 25, 29, 40), yet "initial" sorption of *S. mutans* has been shown to occur in the absence of sucrose (4). *S. mutans* dependency on sucrose to form plaque has been confirmed in this study for Bratthall serotypes *a*, *b*, and *d*. However, both initial attachment and subsequent in vitro accumulation of several serotype *c* and *e* strains has not been shown to be obligatorily sucrose dependent, although tenacity of accumulation appears to be fostered by sucrose.

The ability to form an adherent plaque in vitro and in vivo and to cause disease has been stated to be dependent on synthesis of alkali-soluble, cell-associated glucans (9, 41). Previous linkage analyses of similar water-insoluble glucan fractions termed "mutan" (15) have demonstrated an abundance of 1 → 3 linkages and branches (8, 15, 16, 19, 32). These studies have involved both serotype *c* and *d* strains. Comparable findings were obtained for adherent, sucrose-cultured cells of NCTC 10449 in this study. Glucans have also been reported to contain secondary 1 → 2 and 1 → 4 linkages (reviewed in 37), with trimethyl glucitol retention times of 1.83 and 2.32, respectively (23). It has been suggested (37) that some of the 1 → 4 linkages may be attributable to intracellular glycogen (intracellular polysaccharide) contaminants. However, we observed no gas-liquid chromatographic peak corresponding to 2,3-dimethyl glucitol (T = 4.50), the derivative of glycogen branch residues, nor was there a shoulder on the peak corresponding to 1 → 6 linkages (T = 2.22). It is not possible to distinguish 1 → 2 from 1 → 3 linkages because of the similarity of the retention times of their trimethyl glucitols, 1.83 and 1.82, respectively. Others have reported that this linkage is a minor or absent secondary linkage (1, 6, 31, 37) in *S. mutans* glucan. Nonetheless, it is possible that the glucans described here contain some 1 → 2 as well as minor amounts of 1 → 4 linkages. Furthermore, based on previous reports (6, 15, 32, 37) we consider these glucose polymers to be joined via α-D-glucopyranosyl linkages.

Recent in vivo data in rat caries models show that the minimal colonizing dose of *S. mutans* for Sprague-Dawley rats is not dramatically different in the presence of sucrose- or glucose-containing diets when the infectant is a serotype *c* representative (47). However, for serotype *d* strains, the minimum colonizing dose of *S. mutans* is much lower when animals are sucrose fed than when they are glucose fed (50, 51). Additionally, *S. mutans* has been found to colonize the teeth of sucrase-deficient children who consume negligible amounts of sucrose (48). Further interpretation of these in vivo studies may now be possible in light of our data, which show that bacterial attachment and accumulative growth by some *S. mutans* strains on smooth surfaces are not sucrose dependent.

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