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The Role of Sucrose in Cariogenic Dental Biofilm Formation—New Insight

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Abstract

Dental caries is a biofilm-dependent oral disease, and fermentable dietary carbohydrates are the key environmental factors involved in its initiation and development. However, among the carbohydrates, sucrose is considered the most cariogenic, because, in addition to being fermented by oral bacteria, it is a substrate for the synthesis of extracellular (EPS) and intracellular (IPS) polysaccharides.

Therefore, while the low pH environment triggers the shift of the resident plaque microflora to a more cariogenic one, EPS promote changes in the composition of the biofilms' matrix. Furthermore, it has recently been shown that the biofilm formed in the presence of sucrose presents low concentrations of Ca, P_i, and F, which are critical ions involved in de- and remineralization of enamel and dentin in the oral environment. Thus, the aim of this review is to explore the broad role of sucrose in the cariogenicity of biofilms, and to present a new insight into its influence on the pathogenesis of dental caries.

Keywords

biofilm; sucrose; cariogenic

INTRODUCTION

Dental caries results from the interaction of specific bacteria with constituents of the diet within a biofilm termed 'dental plaque' (Bowen, 2002). Sucrose is considered the most cariogenic dietary carbohydrate, because it is fermentable, and also serves as a substrate for the synthesis of extracellular (EPS) and intracellular (IPS) polysaccharides in dental plaque (Newbrun, 1967; Bowen, 2002).

Thus, low pH induced by sucrose fermentation triggers a shift in the balance of resident plaque microflora to a more cariogenic one, according to the ecological plaque hypothesis (Marsh, 1991). This hypothesis has been supported by long-term dietary sugar consumption (De Stoppelaar *et al.*, 1970; Dennis *et al.*, 1975; Staat *et al.*, 1975) and *in situ* experimental studies (Minah *et al.*, 1981; Pecharki *et al.*, 2005; Ribeiro *et al.*, 2005).

Furthermore, EPS (mainly insoluble glucans) promote bacterial adherence to the tooth surface (Rölla, 1989; Schilling and Bowen, 1992) and contribute to the structural integrity of dental biofilms. They also increase the porosity of biofilm formed, allowing sugar to diffuse into the deepest parts of the biofilm (Dibdin and Shellis, 1988), which would result in low plaque pH

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values, due to microbial catabolism (Zero *et al.*, 1986b). There is also evidence showing that sucrose exposure and insoluble EPS are associated with the pathogenesis of dental caries (Johnson *et al.*, 1977; Zero *et al.*, 1986b; Cury *et al.*, 1997, 2000; Mattos-Graner *et al.*, 2000; Nobre dos Santos *et al.*, 2002; Paes Leme *et al.*, 2004; Pecharki *et al.*, 2005; Ribeiro *et al.*, 2005; Aires *et al.*, 2006).

Therefore, it is clear that EPS are critical virulence factors in the dental biofilm formed in the presence of sucrose (Bowen, 2002). The relationship between EPS and caries has been supported by *in situ* and clinical studies, and, simultaneously, it has been found that sucrose reduces the concentrations of calcium (Ca), inorganic phosphorus (P_i), and fluoride (F) in the dental biofilm (Cury *et al.*, 1997, 2000, 2003; Nobre dos Santos *et al.*, 2002; Paes Leme *et al.*, 2004; Pecharki *et al.*, 2005; Ribeiro *et al.*, 2005; Aires *et al.*, 2006).

Ca, P_i , and F are ions that are important in maintaining the mineral equilibrium between the tooth and the oral environment (Margolis *et al.*, 1988; Pearce, 1998). Low pH and the concentrations of these ions are critical factors during the de- and remineralization processes in the saliva/biofilm/teeth milieu (Pearce, 1998), and the reduction of ion availability may increase the cariogenic potential of the biofilm (Margolis and Moreno, 1992; Cury *et al.*, 1997, 2000, 2003; Gao *et al.*, 2001; Ribeiro *et al.*, 2005; Aires *et al.*, 2006). However, the exact mechanisms by which sucrose reduces the inorganic content in the matrix of the cariogenic biofilm remain to be elucidated.

Some hypotheses have been suggested to explain how sucrose changes the inorganic concentrations in biofilms: (1) Constant low-pH values attained in the biofilm matrix, due to persistent sucrose fermentation, would dissolve mineral reservoirs or obviate their storage; (2) enamel could take up these ions from dental biofilm fluid; (3) the low pH values caused by sucrose fermentation in biofilms would release the reservoir of ions bound to bacterial cell walls; (4) low bacterial density due to high insoluble EPS content could result in fewer binding sites for these ions; and (5) low concentrations of specific ion-binding proteins could result in fewer mineral reservoirs in biofilms formed in the presence of sucrose.

Thus, the aim of this review is to consider the broad role of sucrose in the cariogenic properties of the biofilm, and to discuss tenable hypotheses to explain the low inorganic ion concentrations found in the matrix of the biofilms formed in the presence of this carbohydrate.

(1) THE “ECOLOGICAL PLAQUE HYPOTHESIS” AND DENTAL PLAQUE AS A BIOFILM

The ecological plaque hypothesis was proposed in an attempt to unify some of the clinical and laboratory observations (Theilade, 1986; Marsh, 1991) by combining elements of the non-specific (Theilade, 1986) and the specific (Loesche, 1976) theories of the relationship between dental plaque and dental diseases. Thus far, it is the best explanation for the microbial etiology of dental diseases (Theilade, 1996).

With regard to dental caries, and according to this hypothesis, a change in a key environmental factor will trigger a shift in the balance of the resident plaque microflora, which would promote the emergence of more cariogenic bacteria and change the equilibrium toward dental demineralization (Marsh, 1994) (Fig. 1A). Dietary fermentable carbohydrates have been recognized as primary factors responsible for biochemical and physiological changes in dental biofilms. It is well-established that, after the intake of fermentable sugars (glucose, sucrose, or fructose), the pH in plaque falls rapidly, from around neutrality to pH 5.0 or below (Stephan, 1944; Bowen *et al.*, 1966). In addition, frequent long-term carbohydrate consumption increases the proportions of mutans streptococci and lactobacilli, with a concomitant decrease in levels

of *S. sanguinis* and other oral streptococci (De Stoppelaar *et al.*, 1970; Dennis *et al.*, 1975; Staat *et al.*, 1975). However, it was unclear whether the increase in the levels of cariogenic bacteria was due to availability of sugar *per se* or a response to persistent low pH following constant sugar catabolism (Marsh, 2003). Since these two conditions cannot be distinguished *in vivo*, Bradshaw *et al.* (1989) demonstrated *in vitro* that a decrease of the environmental pH after a glucose pulse increased the percentage of viable counts of *S. mutans* and *L. casei* by 19 and 180 times, respectively, as compared with the counts at a constant pH of 7.0. Subsequently, it was shown that mutans streptococci or lactobacilli are competitive at the low pH values attained within biofilms, which inhibits the growth and metabolism of non-cariogenic species (Bradshaw and Marsh, 1998). Collectively, these *in vitro* studies showed that the breakdown of microbial homeostasis in dental biofilms is caused by low pH generated from carbohydrate metabolism, rather than by carbohydrate availability. The survival of specific bacteria is mainly due to their acid tolerance/adaptation mechanisms within biofilms (Zero, 1993; Burne, 1998; Quivey *et al.*, 2000). In addition, the ecological plaque hypothesis has been supported by *in situ* studies, which showed a clear relationship between sugar exposure and increase of mutans streptococci and lactobacilli in dental biofilms formed (Pecharki *et al.*, 2005; Ribeiro *et al.*, 2005).

Nevertheless, the low pH generated by sugar metabolism and the subsequent shifts in microbial composition may not be the only factor involved in the pathogenesis of dental caries. A recent *in situ* study reported that biofilms formed on enamel by frequent exposure to starch displayed numbers of lactobacilli 200 times higher compared than those formed in the absence of the sugar, but this factor was not enough to induce significant enamel mineral loss (Ribeiro *et al.*, 2005). In contrast, enamel demineralization was detected when the biofilm was formed in the presence of sucrose, which induced similar increases in the levels of aciduric bacteria.

Therefore, factors other than acidogenicity may explain the distinct cariogenic potentials among carbohydrates (Carlsson and Egelberg, 1965; Krasse, 1965; Edwardsson and Krasse, 1967; Carlsson and Sundström, 1968; Birkhed *et al.*, 1980; Lingström *et al.*, 1994; Mattos-Graner *et al.*, 1998; Cury *et al.*, 2000; Ribeiro *et al.*, 2005).

(2) THE ROLE OF SUCROSE IN BIOFILM CARIOGENICITY

There is a clear causal relationship between sucrose and dental caries that has been demonstrated in both epidemiological and experimental studies (Edwardsson and Krasse, 1967; Birkhed *et al.*, 1980; Downer, 1999; Cury *et al.*, 1997, 2000, 2001; Nobre dos Santos *et al.*, 2002; Zero, 2004, and references therein). Sucrose causes major biochemical and physiological changes during the process of biofilm formation, which, in turn, enhance its caries-inducing properties.

Sucrose promotes an increase in the proportions of mutans streptococci and lactobacilli and, simultaneously, a decrease in *S. sanguinis* levels as a result of the pH fall caused during the fermentation of this carbohydrate (De Stoppelaar *et al.*, 1970; Dennis *et al.*, 1975; Staat *et al.*, 1975; Minah *et al.*, 1981). This observation suggests that acid production from sucrose metabolism disrupts the balance of the microbial community, favoring the growth of cariogenic species (Marsh, 1991). Recent studies have showed that biofilms formed in the presence of sucrose displayed lower fasting and final pH levels, higher mutans streptococci and lactobacilli counts, and enhanced cariogenicity than did those formed in the absence of the sugar (Pecharki *et al.*, 2005; Ribeiro *et al.*, 2005). In addition, the cariogenicity of sucrose has been associated with its frequency of exposure and concentration (König *et al.*, 1968; Hefti and Schmid, 1979; Bowen *et al.*, 1980; Cury *et al.*, 1997; Duggal *et al.*, 2001; Paes Leme *et al.*, 2004; Aires *et al.*, 2006). An increase in the frequency of exposure to carbohydrates results in the plaque being subjected to a prolonged period below the critical pH for enamel demineralization,

whereas a greater decrease in pH is observed when sucrose concentration increases. These latter conditions would favor the growth and selection of cariogenic bacteria, thereby converting a healthy biofilm to a diseased one, and consequently enhancing demineralization (Marsh, 1991). This suggests that sucrose may act as a typical fermentable carbohydrate source; however, in comparison with other carbohydrates, sucrose shows enhanced cariogenicity (Bowen *et al.*, 1966; Edwardsson and Krasse, 1967; Birkhed *et al.*, 1980; Horton *et al.*, 1985; Cury *et al.*, 2000; Ribeiro *et al.*, 2005).

Furthermore, two recent *in situ* studies clearly demonstrated that sucrose has additional properties that enhance its cariogenic potential in comparison with glucose and fructose (Cury *et al.*, 2000) or starch (Ribeiro *et al.*, 2005). For example, sucrose promoted higher enamel mineral loss when compared with a mixture of equimolar concentrations of glucose and fructose (Cury *et al.*, 2000). Sucrose also induced lower pH values, higher mutans streptococci counts in biofilm, and higher mineral loss when compared with starch alone. Indeed, when sucrose and starch were used in combination, the cariogenic potential of starch was enhanced (Ribeiro *et al.*, 2005).

Thus, sucrose is a unique cariogenic carbohydrate, because it is fermentable and also serves as a substrate for extracellular glucan synthesis by glucosyltransferases (GTFs) from mutans streptococci (Newbrun, 1967; Bowen, 2002). Several studies have showed a direct relationship between sucrose exposure and EPS, and caries development (Fig. 1B) (Johnson *et al.*, 1977; Cury *et al.*, 1997, 2000; Mattos-Graner *et al.*, 2000; Nobre dos Santos *et al.*, 2002; Pecharki *et al.*, 2005; Ribeiro *et al.*, 2005).

(3) POLYSACCHARIDE ENHANCES THE CARIOGENICITY OF BIOFILMS

The polysaccharides in biofilms can be divided into two categories: (1) extracellular polysaccharides (EPS), which promote bacterial accumulation to the tooth surface, and influence the physical and biochemical properties of biofilms; and (2) intracellular polysaccharides (IPS), which serve as an endogenous source of carbohydrates that can be metabolized to produce acids during periods of nutrient limitation (Tanzer *et al.*, 1976; Zero *et al.*, 1986a). Both EPS and IPS have important roles in the cariogenicity of biofilms, as discussed below.

Extracellular Polysaccharides (EPS)

Using sucrose primarily as a substrate, the EPS are synthesized mostly by bacterial glucosyltransferases (GTFs) and, to a lesser extent, by fructosyltransferases (FTFs) (Hamada and Slade, 1980; Bowen, 2002). The GTFs from *S. mutans* synthesize a mixture of $\alpha(1\rightarrow3)$ -linked insoluble glucans and $\alpha(1\rightarrow6)$ -linked soluble glucans, whereas FTF produces $\alpha(2\rightarrow6)$ -linked fructans. The EPS are largely insoluble, have a complex structure (Kopeck *et al.*, 1997), and promote selective adherence (Schilling and Bowen, 1992; Vacca-Smith *et al.*, 1996) and accumulation of large numbers of cariogenic streptococci on the teeth of human subjects (Rölla, 1989; Mattos-Graner *et al.*, 2000; Nobre dos Santos *et al.*, 2002) and experimental animals (Krasse, 1965; Frostell *et al.*, 1967; Johnson *et al.*, 1977). Furthermore, EPS increase the bulk and porosity of dental plaque matrix, thereby allowing more substrate to diffuse to the enamel surface (Dibdin and Shellis, 1988). As a result of enhanced substrate diffusibility, deeper layers of dental plaque display lower pH values, due to sugar metabolism by acidogenic micro-organisms (Zero *et al.*, 1992), thereby enhancing the development of dental caries (Cury *et al.*, 1997, 2000; Mattos-Graner *et al.*, 2000; Nobre dos Santos *et al.*, 2002; Ribeiro *et al.*, 2005). A recent study with various mutants constructed by allelic exchange in the regions coding for GTFs and FTFs showed that the wild-type strain produced larger quantities of water-insoluble glucan and allowed faster diffusion of hydrogen ions, compared with mutants (Hata and Mayanagi, 2003).

The relationship among sucrose exposure, EPS, and caries development has been demonstrated in several studies. For example, mutant strains of *S. mutans* defective in the *gtf* genes, especially *gtfB* and *gtfC*, are significantly less cariogenic than are the parent strains in animals (Johnson *et al.*, 1977; Yamashita *et al.*, 1993). *In situ* studies have showed that a higher concentration and frequency of sucrose exposure increased EPS concentration in the biofilm matrix, lowered fasting pH values, and enhanced enamel demineralization when compared with biofilms formed in the absence of sucrose (Cury *et al.*, 1997, 2000; Ribeiro *et al.*, 2005; Aires *et al.*, 2006) (Table). Furthermore, clinical studies have also suggested that synthesis of EPS is related to caries activity in children (Mattos-Granner *et al.*, 2000; Nobre dos Santos *et al.*, 2002) (Table). It is evident that sucrose and GTFs are key factors involved in the synthesis of these complex polysaccharides.

However, other factors may influence the biochemistry and structural integrity of EPS. A recent *in situ* study showed that biofilms formed in the presence of sucrose and starch were more cariogenic than those exposed to sucrose alone, despite the fact that the total amounts of EPS in the biofilm matrices were similar in these two conditions (Ribeiro *et al.*, 2005). However, the formation of glucans and the adherence of oral micro-organisms can be modulated by the interaction of amylase and GTF enzymes adsorbed onto the hydroxyapatite surface (Vacca-Smith *et al.*, 1996), which may influence the formation and cariogenicity of dental biofilms.

Furthermore, there is evidence showing that the structure of glucans could be influenced by glucanohydrolases present in the oral cavity. For example, the presence of dextranase and/or mutanase during glucans synthesis by GTFs caused linkage remodeling and branching, which influenced the bacterial binding sites on these glucans (Hayacibara *et al.*, 2004). It is noteworthy that while the synthesis of polysaccharides by plaque bacteria during sucrose-rich diet increases, the levels of dextranase and levanase of plaque bacteria also increase (Gawronski *et al.*, 1975). The presence of glucanohydrolases may have an impact on the development, physical properties, and bacterial binding sites of the polysaccharide matrix in dental biofilms.

Clearly, EPS play a major role in the pathogenesis of dental caries, by promoting biochemical and physiological changes in the matrix of the biofilm, including: (i) enhancing bacterial adherence and further accumulation of organisms, (ii) providing structural integrity and bulk to biofilms, and (iii) increasing the acidogenicity of the biofilm matrix.

Intracellular Polysaccharides (IPS)

IPS are high-molecular-weight metabolizable glycogen-like storage polymers with $\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow6)$ linkages, which provide the micro-organisms with an endogenous source of carbohydrate during periods of nutrient limitation in the oral cavity (Hamilton, 1976; Tanzer *et al.*, 1976). Consequently, IPS can promote the formation of dental caries by prolonging the exposure of tooth surfaces to organic acids and maintaining a lower fasting pH in the matrix of the plaque (Tanzer *et al.*, 1976). It is noteworthy that an *S. mutans* mutant that synthesizes elevated levels of IPS was significantly more cariogenic in animals than was the wild-type (Harris *et al.*, 1992; Spatafora *et al.*, 1995).

The importance of IPS for *S. mutans* virulence supports previous reports in the literature showing an association of these storage polysaccharides with dental caries in animals and in humans (Gibbons and Socransky, 1962; Loesche and Henry, 1967; van Houte *et al.*, 1969; Tanzer *et al.*, 1976; Ashley and Wilson, 1977; Zero *et al.*, 1986a; Spatafora *et al.*, 1995).

Furthermore, *in situ* studies have showed that acid production by *S. mutans* from endogenous substrate caused pronounced and prolonged decreases in pH, and enhanced enamel demineralization (Zero *et al.*, 1986a; van Houte *et al.*, 1989). Recently, it was observed that biofilm formed *in situ* in the presence of 20% (or higher) sucrose showed a significantly lower

fasting pH compared with a negative control (Pecharki *et al.*, 2005; Ribeiro *et al.*, 2005; Aires *et al.*, 2006), which could be related to the metabolism of IPS. In addition, biofilms formed *in situ* in the presence of glucose+fructose and sucrose displayed higher concentrations of IPS than those formed in the absence of these carbohydrates (Tenuta *et al.*, 2006). Interestingly, the differences in IPS concentration were not only maintained during 3, 7, and 14 days of biofilm formation, but also increased over time (unpublished data). Thus, the metabolism of IPS could explain the fasting low pH observed in biofilms and, consequently, the increased cariogenicity of these biofilms.

Collectively, these observations indicate that the metabolism of endogenous substrate is a significant trait in the pathogenesis of dental caries by influencing the acidogenicity and resting pH of dental biofilm.

Thus, there is clear evidence that EPS and IPS influence the cariogenicity of dental biofilms by at least two pathways: (1) EPS promote bacterial adherence and accumulation on tooth surfaces, and cause biochemical and structural changes in the matrix of the biofilms; and (2) IPS promote lower fasting pH levels during periods of nutrient deprivation, which could result in the selection of cariogenic micro-organisms and caries development. Recently, it was shown that therapeutic agents that diminish EPS and IPS concentrations in biofilms also reduce the development of dental caries in rats (Koo *et al.*, 2003, 2005), confirming the importance of these polysaccharides in *S. mutans* cariogenicity.

(4) EPS MAY CHANGE THE INORGANIC COMPOSITION OF BIOFILMS

Among the chemical changes that may be associated with the presence of EPS, the low concentrations of Ca, P_i, and F are the most intriguing and a relevant factor in the context of biofilm cariogenicity. The low concentrations of ions are directly related to the saturation levels of biofilm, which determines the driving force of minerals for the demineralization process (Pearce, 1998).

The concentrations of Ca and P_i in dental plaque are critical in terms of caries development, because there is an inverse relationship between concentrations of these ions in the plaque matrix (Ashley and Wilson, 1977) and fluid (Margolis and Moreno, 1992), and caries experience. These ions would be released to the plaque/enamel interface during the fall of pH, thereby maintaining the aqueous phase in a saturated condition.

In situ studies have showed a relationship between sucrose and increased concentrations of EPS and, simultaneously, reduced Ca, P_i, and F content in the matrix of dental plaque, which enhanced tooth enamel mineral loss (Cury *et al.*, 1997, 2000; Paes Leme *et al.*, 2004; Pecharki *et al.*, 2005; Aires *et al.*, 2006) (Table). Furthermore, a recent study showed that biofilms formed in the presence of sucrose, alone or in combination with starch, displayed inorganic concentrations lower than those formed either in the absence of sugar or in the presence of starch only, and which resulted in higher enamel demineralization (Ribeiro *et al.*, 2005). Nobre dos Santos *et al.* (2002) also found lower concentrations of F, Ca, and P_i in dental plaque samples collected from nursing children with caries, when compared with those from caries-free children. Whether the low concentrations of Ca, P_i, and F are also detected in the fluid of the biofilms needs to be determined in further studies.

Nevertheless, the findings suggest that sucrose, alone or in combination with other carbohydrates, is associated with lower inorganic ion concentrations found in the matrix of dental biofilm, thereby augmenting its cariogenicity (Cury *et al.*, 2000; Ribeiro *et al.*, 2005) (Table). Furthermore, it is likely that the concentrations of inorganic ions are related to EPS content in dental biofilm matrix, but the exact mechanisms of how this phenomenon occurs remain to be elucidated. Therefore, we propose five hypotheses and present some experimental

evidence to identify a plausible explanation for the lower inorganic concentrations in cariogenic biofilms.

(5) HOW CAN THE LOW INORGANIC ION CONCENTRATION IN A CARIOGENIC BIOFILM BE EXPLAINED?

Recent studies showing that dental biofilm formed in the presence of sucrose displayed low inorganic ion concentrations in the biofilm matrix (Table) (Cury *et al.*, 1997;2000;2003;Paes Leme *et al.*, 2004;Ribeiro *et al.*, 2005;Aires *et al.*, 2006) provide new insight into a better understanding of the pathogenesis of development of cariogenic dental biofilms. Thus, some hypotheses based on the structure, composition, and ion kinetics of biofilms may explain the possible mechanisms for the lower inorganic ion concentrations in the presence of carbohydrates.

(1) Depletion of Mineral Reservoirs

The first hypothesis proposes that the constant low pH, due to sucrose fermentation, would release ions from mineral deposits (Pearce, 1998), which could then diffuse into saliva, resulting in a biofilm with low inorganic concentration. Another alternative explanation is that constant low pH maintained in the biofilm would prevent the precipitation of minerals (Tenuta *et al.*, 2006). However, dental plaque samples in our studies were collected 10-12 hrs after the last sucrose exposure (Cury *et al.*, 1997, 2000, 2003; Paes Leme *et al.*, 2004; Ribeiro *et al.*, 2005; Aires *et al.*, 2006), which would have been enough time for the mineral ions that had been lost to saliva to be replaced by the simple law of mass action. Furthermore, the concentrations of Ca, P_i, and F in dental biofilm formed in the absence or presence of sucrose neither decreased nor increased after sucrose exposure was provided (for biofilms formed in its absence) or interrupted (for biofilms formed in its presence) for a further 48 hrs (Cury *et al.*, 2003). Thus, the inorganic ion concentrations in biofilm may be attributed to changes in the matrix structure, rather than to depletion of inorganic pools by organic acids (Cury *et al.*, 2003) (Fig. 2).

(2) Enamel Uptake of Ions from Biofilm Fluid

The depletion of ions in the biofilm matrix could be a result of their uptake by enamel, since, during falls in pH, the biofilm fluid would be undersaturated relative to hydroxyapatite, but would be still oversaturated relative to fluorapatite, which precipitates on the enamel (Larsen, 1990). This observation could explain the reduction of F concentration in biofilm, but it would not explain the simultaneous low concentrations of Ca and P_i found in our studies (Fig. 2). Furthermore, the mineral ions taken up by enamel could be replaced, since the plaque samples were collected 12 hrs after the last sucrose exposure.

(3) Release of Ions Bound to Bacterial Cells

Another hypothesis is based on the ability of bacterial cell walls to bind ions, which could act as a reservoir of ions in dental plaque (Fig. 2). For example, calcium binding in streptococci is predominantly phosphate-group-based, whereas that in *L. casei* and *A. naeslundii* is predominantly carboxylate-group-based (Rose *et al.*, 1997a). This reservoir of ions could explain our findings on not only Ca concentrations in the matrix of the biofilm, but also on fluoride, since Zn²⁺, Mg²⁺, and Ca²⁺ at 5 mmol/L enhance fluoride binding to the cell wall (Rose *et al.*, 1996). These reservoirs are physicochemically sensitive to pH changes, which means that they are depleted during the falls in pH, but would be replenished when the pH rises again. However, this phenomenon would not explain the low concentration of P_i that has been found in dental biofilm formed in the presence of sucrose (Cury *et al.*, 1997,2000, 2003;Pecharki *et al.*, 2005;Ribeiro *et al.*, 2005;Aires *et al.*, 2006). Furthermore, the low

inorganic ion concentrations have been found 12 hrs after the last exposure to sucrose, which would be enough time for this reservoir to be replenished. Therefore, this hypothesis may not be the best explanation for the low concentrations of Ca, P_i, and F found in dental biofilm formed in the presence of sucrose.

(4) Low Density of Bacteria

In contrast, the concept of bacterial binding sites would be extremely important, considering the density of bacteria in biofilm (Carlsson and Sundström, 1968) (Fig. 2), which could be influenced by the amount of insoluble EPS. These polysaccharides occupy a large volume of dental plaque, thereby reducing the number of bacteria and, consequently, ion-binding sites. When the frequency of sucrose exposure was increased, a higher concentration of EPS (Cury *et al.*, 1997; Pearce *et al.*, 2002) and lower cell biomass content in biofilm (Pearce *et al.*, 2002) were observed. Although this hypothesis could explain the low concentrations of Ca (Rose *et al.* (1997b) and F (Rose *et al.*, 1996), it does not elucidate the simultaneous decrease of P_i.

(5) Low Concentrations of Specific Proteins

The last proposed hypothesis to explain the simultaneous low concentrations of Ca, P_i, and F would be the protein composition of the dental plaque matrix (Fig. 2). Analyses of recent data showed clear differences in the patterns of the matrix proteins extracted from dental plaque formed under three distinct conditions: (1) in the absence of sugar (control), (2) in the presence of glucose and fructose, and (3) in the presence of sucrose (Cury *et al.*, 2000). In terms of the protein profiles and their concentrations in the biofilms, it would be relevant if there were differences in their ability to bind calcium and provide a template for mineral growth.

Recently, it was shown that approximately 33% of the total calcium in dental plaque fluid is free, 17% is bound to phosphate and organic acid anions, and 50% is bound to other species (such as proteins) (Gao *et al.*, 2001). If proteins are responsible for 50% of the calcium concentration of plaque, a change in protein profile could affect calcium-binding sites. This observation may explain the findings that biofilm formed in the presence of sucrose exhibited lower inorganic ion concentrations (Cury *et al.*, 1997, 2000, 2003; Paes Leme *et al.*, 2004; Pecharki *et al.*, 2005; Ribeiro *et al.*, 2005). Whether calcium-binding proteins from saliva or from bacteria can actually serve as a template for mineral growth in dental biofilms awaits further evaluation.

Proline-rich proteins (PRP), statherin, histatins identified in acquired enamel pellicle (Schüpbach *et al.*, 2001), cysteine-containing phosphoproteins in dental plaque (DiPaola *et al.*, 1984), and low-molecular-weight peptides in human parotid saliva (Perinpanayagam *et al.*, 1995) may play significant roles as calcium-binding proteins. A study of the calcium-binding properties of acidic PRP indicated that there is interaction between the calcium-binding N-terminal end and the proline-rich C-terminal (Bennick, 1987). PRP and statherin are also potent inhibitors of calcium phosphate precipitation (Moreno *et al.*, 1979). Moreover, the low-molecular-weight peptides are likely to be in equilibrium with dental plaque fluid, and may therefore help modulate events such as demineralization and remineralization, microbial attachment, and dental plaque metabolism at the tooth-saliva interface (Perinpanayagam *et al.*, 1995). These proteins bind preferentially to hydroxyapatite surfaces, and may bind to calcium. The protein-binding mechanism could be similar to that of casein phosphopeptides (CPP), a protein that stabilizes amorphous calcium phosphate (ACP) to form small clusters, which are able to release calcium to inhibit demineralization and/or enhance remineralization (Rose, 2000). The addition of CPP-ACP to either sorbitol- or xylitol-based sugar-free gum resulted in a dose-dependent increase in enamel subsurface remineralization (Shen *et al.*,

2001). Therefore, the calcium-binding proteins can work as a calcium reservoir and modulate crystal growth, and thus interfere with the de-/remineralization of dental enamel.

Several studies have identified calcium-binding proteins in saliva, acquired pellicle, and gingival crevicular fluid by using two-dimensional gel electrophoresis (2D-PAGE) and peptide mass fingerprinting (Kojima *et al.*, 2000; Ghafouri *et al.*, 2003; Yao *et al.*, 2003; Huang, 2004). Nevertheless, none of them analyzed the protein profile in the matrix of dental biofilms. The protein profiles in biofilms formed in the absence or presence of sucrose were recently evaluated by 2D-PAGE (Paes Leme *et al.*, 2003) and peptide mass fingerprinting (Paes Leme *et al.*, 2004) (Figs. 3A, 3B). Calcium-binding proteins were identified only in biofilm formed in the absence of sucrose (Paes Leme *et al.*, 2004) (Fig. 3A). This finding is the first evidence showing that the absence of calcium-binding proteins in biofilm formed in the presence of sucrose may be associated with the low concentration of calcium in its matrix, which would promote conditions of undersaturation and, consequently, favor the demineralization process.

The qualitative differences in the protein composition of dental biofilm formed in the presence of sucrose may be related to elevated levels of EPS in the matrix; these polysaccharides occupy a large volume of the biofilm, which decreases the binding sites for proteins. Moreover, it is not known whether the presence of ions, such as calcium, is necessary for protein binding, or if these specific proteins might serve as a template for mineral-binding sites.

The findings of undetectable levels of calcium-binding proteins in biofilm formed in the presence of sucrose offer the most promise among the different hypotheses discussed here, and could be one of the pathways by which this carbohydrate influences the cariogenicity of biofilms.

(6) CONCLUSION

The low concentrations of Ca, P_i, and F in the matrix of the whole dental biofilm formed in the presence of sucrose can be an additional factor contributing to the cariogenicity of this carbohydrate. However, the explanation of these findings remains to be elucidated. It is clear that a better understanding of the unique cariogenic properties of this dietary carbohydrate at physicochemical and molecular levels is worthy of additional exploration.

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Figure 1. Schematic illustration of a cariogenic biofilm formation in the presence of fermentable sugars (**A**) or in the presence of sucrose (**B**); EPS enhances the cariogenic potential of the biofilm formed in the presence of sucrose (adapted from Marsh, 1994).

Figure 2.

Schematic representation of the first, second, third, fourth and fifth hypotheses, respectively. **First hypothesis:** Constant low pH caused by sucrose fermentation would release ions from mineral deposits, which could diffuse to saliva, and promote dental plaque with lower inorganic ion concentrations. However, after 10-12 hrs, there would have been enough time for the mineral ions, which had been lost to saliva, to be replaced by the simple law of mass action. **Second hypothesis:** Enamel could have taken up ions from biofilm fluid during pH-cycling. After 12 hrs, the biofilms would have been again saturated with these ions. **Third hypothesis:** Schematic representation adapted from Rose *et al.* (1996). Binding to bacterial cell wall is another reservoir of minerals. When the pH falls, the minerals are released from biofilm. After the pH is increased, the biofilm is saturated again with the ions from saliva. **Fourth hypothesis:** Bacterial density. Note that, in the second circle, the density of bacteria is lower, since polysaccharides occupy a large volume of the biofilm. **Fifth hypothesis:** Low concentrations of specific ion-binding proteins. It has been suggested that biofilm formed in the presence of sucrose shows fewer calcium-binding sites for proteins.

Figure 3.

Two-dimensional gel electrophoresis of dental biofilm formed in the absence (A) and presence (B) of sucrose (20 μ g of proteins). Isoelectric focusing with pH range 4-7 and PAGE (8-18%). The gels were silver-stained. Spots were excised for in-gel digestion and analyzed by mass spectrometry (MALDITOF). The fingerprints were analyzed in Mascot and Prospector program with the NCBI database. Arrows show calcium-binding proteins present only in biofilm formed in the absence of sucrose (A) when compared with biofilm formed in the presence of sucrose (B).

Table

Biofilm Analyses and Enamel Demineralization from Previously Published Studies

Paper	Treatment	F, $\mu\text{g/g}^3$	Ca, mg/g^3	Biofilm Concentrations P, mg/g^3	EPS, mg/g^3	SMH Change (%) ⁴	Mineral Loss ΔZ^5
Cury <i>et al.</i> , 1997 ¹	Sucrose 0	18.7 $\pm$ 3.3 ^c	12.9 $\pm$ 2.5 ^b	3.7 $\pm$ 1.1 ^b	12.0 $\pm$ 2.6 ^b		
	Sucrose 2x	9.6 $\pm$ 2.8 ^b	4.0 $\pm$ 1.0 ^a	0.8 $\pm$ 0.2 ^a	11.5 $\pm$ 1.8 ^b		
	Sucrose 4x	2.7 $\pm$ 0.8 ^a	4.0 $\pm$ 1.1 ^a	0.4 $\pm$ 0.1 ^a	14.0 $\pm$ 3.3 ^b		--
	Sucrose 8x	2.7 $\pm$ 0.4 ^a	4.2 $\pm$ 1.1 ^a	0.4 $\pm$ 0.04 ^a	35.8 $\pm$ 6.9 ^a		
Cury <i>et al.</i> , 2000 ¹	Control	140.6 $\pm$ 30.8 ^a	17.0 $\pm$ 2.8 ^a	11.5 $\pm$ 2.1 ^a	6.5 $\pm$ 1.0 ^a	- 3.3 $\pm$ 0.7 ^a	227,021.7 $\pm$ 3951.5 ^a
	Glucose 10% + Fructose 10%	27.4 $\pm$ 15.9 ^b	1.9 $\pm$ 0.7 ^b	0.5 $\pm$ 0.1 ^b	11.8 $\pm$ 1.9 ^a	-44.2 $\pm$ 7.2 ^b	20,159.5 $\pm$ 2213.4 ^b
	Sucrose 20%	5.6 $\pm$ 2.2 ^b	0.6 $\pm$ 0.1 ^b	0.3 $\pm$ 0.04 ^b	35.0 $\pm$ 7.8 ^b	-73.4 $\pm$ 6.9 ^c	12,414.4 $\pm$ 2419.4 ^c
Cury <i>et al.</i> , 2001	Sucrose 0						226,949.2 $\pm$ 632.1 ^a
	Sucrose 2x						25,527.6 $\pm$ 785.0 ^a
	Sucrose 4x	--	--	--	--		24,000.8 $\pm$ 1157.5 ^a
	Sucrose 8x						15,887.4 $\pm$ 2739.2 ^b
Nobre dos Santos <i>et al.</i> , 2002 ²	Caries-free	58.1 $\pm$ 21.3 ^a	10.6 $\pm$ 5.4 ^a	6.0 $\pm$ 2.9 ^a	39.2 $\pm$ 7.4 ^a		
	Pit/Fissure Caries Nursing Caries	32.5 $\pm$ 13.6 ^b 6.2 $\pm$ 2.9 ^c	7.9 $\pm$ 4.3 ^a 3.3 $\pm$ 2.6 ^b	4.0 $\pm$ 1.9 ^b 2.6 $\pm$ 1.3 ^b	47.4 $\pm$ 8.9 ^b 55.6 $\pm$ 17.6 ^b		
	Sucrose Exposure	1.1 $\pm$ 0.3 ^a	1.2 $\pm$ 0.7 ^a	0.2 $\pm$ 0.04 ^{a,b}	51.1 $\pm$ 13.6 ^a		
	Sucrose Interruption: 24 hrs 48 hrs	1.6 $\pm$ 0.4 ^a 2.7 $\pm$ 0.6 ^a	1.6 $\pm$ 0.6 ^a 3.0 $\pm$ 1.5 ^a	0.2 $\pm$ 0.1 ^a 0.4 $\pm$ 0.1 ^b	49.7 $\pm$ 11.4 ^a 40.6 $\pm$ 9.6 ^b	--	--
Cury <i>et al.</i> , 2003 ¹	Sucrose Absence	63.3 $\pm$ 23.6 ^a	12.2 $\pm$ 1.9 ^a	4.3 $\pm$ 1.5 ^a	4.6 $\pm$ 0.5 ^a		
	Sucrose Exposure: 24 hrs 48 hrs	86.1 $\pm$ 38.6 ^a 67.9 $\pm$ 37.5 ^a	18.8 $\pm$ 4.5 ^a 14.8 $\pm$ 4.0 ^a	4.9 $\pm$ 1.3 ^a 3.9 $\pm$ 1.1 ^a	10.0 $\pm$ 2.7 ^{a,b} 11.4 $\pm$ 2.3 ^b		
	Sucrose 4x	31.0 $\pm$ 41.7 ^a	0.73 $\pm$ 0.52 ^a	1.0 $\pm$ 0.9 ^a	29.1 $\pm$ 17.5 ^a	-19.7 $\pm$ 17.5 ^a	^d 655.7 $\pm$ 506.2 ^a
	Sucrose 8x	17.3 $\pm$ 28.6 ^b	0.53 $\pm$ 0.37 ^b	0.7 $\pm$ 0.7 ^a	44.3 $\pm$ 25.5 ^b	-29.5 $\pm$ 24.6 ^b	^d 869.8 $\pm$ 726.3 ^b
Pecharki <i>et al.</i> , 2005 ²	Control	352.0 $\pm$ 271.0 ^a	45.9 $\pm$ 27.0 ^a	23.5 $\pm$ 14.2 ^a	35.4 $\pm$ 7.4 ^a	- 5.1 $\pm$ 7.6 ^a	^d 459.4 $\pm$ 212.6 ^a
	20% Sucrose	28.2 $\pm$ 79.0 ^b	2.1 $\pm$ 1.8 ^b	3.0 $\pm$ 1.5 ^b	194.0 $\pm$ 125.0 ^b	-57.3 $\pm$ 32.3 ^b	^d 2,165.5 $\pm$ 1890.3 ^b
Ribeiro <i>et al.</i> , 2005 ²	Control	468.4 $\pm$ 401.8 ^a	45.9 $\pm$ 50.9 ^a	27.1 $\pm$ 29.5 ^a	47.5 $\pm$ 22.8 ^a		^d 447.9 $\pm$ 169.0 ^a
	2% Starch	239.8 $\pm$ 251.3 ^b	17.4 $\pm$ 25.4 ^b	11.6 $\pm$ 14.6 ^b	49.8 $\pm$ 13.5 ^a		^d 420.0 $\pm$ 160.1 ^a
	20% Sucrose	119.9 $\pm$ 179.3 ^c	5.1 $\pm$ 7.9 ^c	4.1 $\pm$ 4.6 ^c	181.6 $\pm$ 115.8 ^b	--	^d 955.6 $\pm$ 543.6 ^b

Paper	Treatment	F, µg/g ³	Ca, mg/g ³	Biofilm Concentrations P _i , mg/g ³	EPS, mg/g ³	SMH Change (%) ⁴	Mineral Loss ΔZ ⁵
Aires <i>et al.</i> , 2006 ²	Starch + Sucrose	55.7 ± 144.3 ^c	4.9 ± 10.5 ^c	3.6 ± 5.0 ^c	201.6 ± 137.6 ^b		^δ 1,421.8 ± 653.8 ^c
	Control	55.1 ± 46.1 ^a	39.4 ± 17.2 ^a	22.0 ± 10.5 ^a	28.1 ± 6.5 ^a	- 3.9 ± 5.0 ^a	^δ 253.0 ± 129.0 ^a
	1% Sucrose	64.0 ± 82.8 ^a	25.1 ± 23.1 ^a	14.9 ± 12.2 ^a	41.6 ± 13.5 ^b	-10.5 ± 14.5 ^a	382.0 ± 224.0 ^a
	5% Sucrose	35.4 ± 53.4 ^{ab}	5.7 ± 5.3 ^b	4.2 ± 3.0 ^b	109.0 ± 85.9 ^c	-48.8 ± 34.4 ^b	796.0 ± 600.0 ^b
	10% Sucrose	11.9 ± 11.9 ^{bc}	4.7 ± 5.1 ^b	3.2 ± 2.2 ^b	102.0 ± 76.0 ^c	-43.8 ± 31.2 ^b	1,050.0 ± 1240.0 ^b
	20% Sucrose	12.3 ± 14.8 ^{bc}	5.4 ± 6.1 ^b	3.7 ± 2.6 ^b	125.0 ± 85.4 ^c	-58.2 ± 32.6 ^b	1,580.0 ± 1540.0 ^{bc}
	40% Sucrose	5.5 ± 2.4 ^c	2.6 ± 1.0 ^b	2.3 ± 0.8 ^b	268.0 ± 163.0 ^d	-65.7 ± 40.1 ^b	1,600.0 ± 862.0 ^c

¹ Wet biofilm basis.

² Dry biofilm basis.

³ Distinct superscript lower-case letters show statistical difference between treatments (p < 0.05).

⁴ Surface microhardness change.

⁵ % vol. min × µm.