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What is This?
Simultaneous Caries Induction and Calculus Formation in Rats  

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Weanling specific pathogen-free Osborne-Mendel rats were fed a high-calcium, high-phosphorus diet with various levels of sucrose and inoculated with Streptococcus sobrinus strain 6715-13WT and Actinomyces viscosus strain OMZ-105 in order to determine whether calculus and caries could develop simultaneously. Rats consumed diets designated RC-16-5, RC-16-25, or RC-16-50 which partially replaced the corn starch component with progressively higher levels of sucrose, thus, to 5, 25, or 50% sucrose. In general, bacterial recoveries of A. viscosus declined with higher sucrose content of the diet, but a pattern of recovery for S. sobrinus was less clear with respect to dietary sucrose. S. sobrinus, however, was recovered at higher percentages from the tooth surface flora at the later two of three sampling dates. Most calculus—identified by the brittle quality, staining characteristics, and apatitic X-ray diffraction patterns of tooth surface deposits—was formed on the maxillary molars, and most carious lesions occurred on mandibular molars. While there was minimal association of the calculus score with the amount of sucrose in the diet, calculus scores increased greatly from 23 to 43 days after infectious challenge. Caries scores, of both fissure and smooth surfaces, by contrast, increased in a dose-response fashion with increasing dietary sucrose and with time. It is thus possible to induce calculus formation and caries simultaneously in specific pathogen-free Osborne-Mendel rats consuming a high-calcium and -phosphorus diet conducive to calculus formation and containing sucrose. This model appears to be well-suited for simultaneous evaluation of the putative calculus-inhibitory and caries-inhibitory effects of oral therapeutic agents.  


Introduction.  

There is a prevalent belief that it is difficult or impossible to induce calculus and caries simultaneously in experimental animals (Baer et al., 1961; Francis and Briner, 1969). While epidemiological data do not strongly suggest an inverse correlation of caries and periodontal disease experience (Miller and Seidler, 1940; Kesel, 1950; White and Russell, 1962), caries scores and calculus scores in some data are inversely correlated. The classic locations of supragingival calculus are not those associated with carious lesions of humans (Alexander, 1971), and populations of humans with extensive calculus accumulations during adolescence and young adulthood have been described as having infrequent carious lesions (Greene, 1960; Ramfjord, 1961). It is a common clinical belief that humans with high caries activity have little tendency to form calculus, and vice versa (Yardeni, 1948; Carranza, 1984). Nonetheless, it is not unusual to find calculus accumulations in some areas of the dentition while finding carious lesions in others (Ainamo, 1970).  

There has been considerable recent interest in calculus-inhibiting dentifrices (ten Cate, 1989) which, it is hoped, retain their caries-inhibitory properties. This has led our laboratory to explore whether it is possible to induce calculus formation and caries simultaneously in an experimental animal model which is amenable to concurrent assessment of anti-calculus and anti-caries effects of topical agents, such as dentifrices and mouthrinses.  

Materials and methods.  

Animal model, diets, inoculations, and experimental design. —Specific pathogen-free Osborne-Mendel (SPFOM) rats of our own animal colony were fed autoclaved, low-fluoride rat chow No. 5010 (Ralston-Purina, St. Louis, MO) and sterile demineralized water ad libitum, and were mated so as to produce sufficient progeny on the same day for subsequent study. The breeding colony was maintained in laminar flow hoods equipped with bacteriological air filters (Portable Containment System-80, Hazelton Systems, Inc., Aberdeen, MD). The colony was demonstrated repeatedly to be free of mutans streptococci by culture of the dentition of its progeny after being fed a diet containing 56% sucrose. For the present study, 54 rats derived from several litters were weaned at 21 days of age, removed from the laminar flow environment, and placed in a room with 13.3 air volume changes per hour and maintained at positive pressure. They were randomly distributed to three large, sterile plastic cages containing autoclaved hardwood chip bedding (Northeastern Products Corp., Warrensburg, NY) and fed one of three modifications of the high-calcium and high-phosphate diet RC-16 (Zeigler Brothers, Gardener's, PA), formulated according to Francis and Briner (1969) (Table). The diet was modified by substitution of powdered sucrose for part of its corn starch such that the final diets contained 5, 25, or 50% (w/w) sucrose (thus termed RC-16-5, RC-16-25, and RC-16-50, respectively) and, conversely, 50, 30, or 5% (w/w) corn starch. Thus, the diets were isocaloric. On day 22 after birth, rats within each diet group were randomly distributed, two per suspended stainless-steel mesh cage (Shor Line No. 5137-1, Schoer Mfg. Co., Kansas City, MO), without bedding. On this and the following day, the rats were orally inoculated with the cariogenic and streptomyein-resistant Streptococcus sobrinus strain 6715-13WT (Tanzier et al., 1974) and with Actinomyces viscosus strain OMZ-105 (supplied by B. Guggenheim). They continued to eat the modified diets and to drink sterile demineral-
ized water, *ad libitum*, for the duration of the study.

Inocula were grown in fluid thioglycollate medium (Difco Laboratories, Detroit, MI) supplemented with 20% (v/v) meat extract (Difco) and excess CaCO<sub>3</sub>. Freshly grown inocula were adjusted to an approximate OD<sub>660nm</sub> of 2.0, and 0.2-mL quantities of both pure cultures were pipetted into the mouth of each rat on two successive days. Spiral plating (Model C Spiral Plater, Spiral Systems, Cincinnati, OH) of appropriate culture dilutions onto trypticase soy agar supplemented with 5% sheep’s blood (BA, Baltimore Biological Labs, Cockeysville, MD) and onto mitis salivarius agar supplemented with potassium tellurite (MS, Difco) revealed that cultures were pure and that total inocula delivered to each rat contained about 10<sup>9</sup> CFU of *S. sobrinus* 6715-13WT and 10<sup>6</sup> CFU of *A. viscosus* OMZ-105, respectively.

At 23 and 43 days post-inoculation, one rat from each cage, and thus half of each diet group, was killed and its dentition further studied as detailed below. Heads were assigned random code numbers, defleshed by dermestid beetles, and scored for caries and calculus deposits, as detailed below. The beetles removed unmineralized plaque from the teeth, as well as soft tissues. Codes were not broken until all scores had been finalized.

*Recovery of micro-organisms.*—At 14, 22, and 42 days post-inoculation, the teeth of all rats were sacrificed for recovery of 6715-13WT and total recoverable flora (Tanzer et al., 1985a, 1985b, 1987) such that 6715-13WT was enumerated on MS supplemented with 200 µg/mL streptomycin sulfate (MSS) and total recoverable flora on BA. Extraneous mutants streptococci or revertants from streptomycin resistance to streptomycin sensitivity were sought on MS; none was observed. *A. viscosus* OMZ-105 and total *A. viscosus* were similarly estimated by microscopic examination of CFU’s on the actinomyces-selective CFAT agar (Zylber and Jordan, 1982). These plates were flooded with 3% H<sub>2</sub>O<sub>2</sub> which allowed for enumeration of the colonies observed to bubble due to the apparent catalase activity of *A. viscosus* (Maiden et al., 1992).

Data were expressed as the percentage of total recoverable flora which was *S. sobrinus* or *A. viscosus*. Other studies had established that percentage recoveries of mutants streptococci from swab samples are proportional to the absolute numbers of mutants streptococci recovered when plaque samples are scraped from molars or upon sonification of the rat’s molar dentition. Absolute counts of only mutants streptococci are potentially misleading, because they reflect the state of cavitation of the teeth more than the mutants colonization level within the tooth’s flora (Tanzer et al., 1982, 1985a,b). Sonification of extracted teeth would also make it difficult for them to be scored for caries and calculus.

*Calculus evaluation and scoring.*—Defleshed specimens were stained for 30 s with 0.024% ammonium purpurate (“murexide”, Sigma Chemical, St. Louis, MO), in a 70% ethanol solution (Pearse, 1985). Deposits were scored on maxillary and mandibular teeth according to the method of Francis and Briner (1969), and dentitions were photographed.

Under a dissecting microscope, deposits were carefully scraped from maxillary teeth of 4 or 5 rats of each diet group, with care taken that teeth not be damaged or tooth materials included. Deposits from individual rats were analyzed by x-ray diffraction with use of a 57.3-µm-diameter Debye-Scherrer powder camera (Philips Electronic Instruments, Mt. Vernon, NY) and CuK<sub>a</sub> radiation. Diffraction patterns were compared with that of a synthetic apatite prepared by hydrolysis of amorphous calcium phosphate at pH 7.4. This latter pattern was typical of apatite with poor crystallinity, such as is found in bone, dentin, and calculus (Rowles, 1964; Schroeder and Bambauer, 1966; Driessens and Verbeeck, 1989).

*Caries scores.*—Carious lesions of mandibular teeth were scored by the method of Keyes (1958b), as modified by Larson (1981), and charted according to the recommendations of Rinehimer and Tanzer (1980).

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**TABLE**

<table>
<thead>
<tr>
<th>EXPERIMENTAL DIETS</th>
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<tr>
<td>Ingredient</td>
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<tr>
<td>Corn Starch</td>
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<td>Nonfat Dry Milk</td>
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<tr>
<td>Liver Powder</td>
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<td>Cellulose</td>
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<td>Cottonseed Oil</td>
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<td>Sucrose (powder)</td>
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<tr>
<td>CaCl&lt;sub&gt;2&lt;/sub&gt;2H&lt;sub&gt;2&lt;/sub&gt;O</td>
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<tr>
<td>NaH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;·H&lt;sub&gt;2&lt;/sub&gt;O</td>
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<tr>
<td>MgSO&lt;sub&gt;4&lt;/sub&gt;</td>
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Diets are modifications of diet RC-16 described by Francis and Briner (1969).

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Fig. 1—Maxillary molar teeth of a rat fed diet RC-16-50. Mineralized deposits (arrows) are stained with murexide and, upon removal from teeth, gave x-ray diffraction patterns illustrated in Fig. 2d. (a) Buccal view; (b) palatal view. Bars represent 1 mm.
Results.

All animals appeared to be well, none died, and groups had statistically indistinguishable weight gains. Fig. 1 illustrates typical deposits remaining on maxillary molar teeth after jaws were defleshed by beetles, suggestive of calculus formation. Incisors were free of such deposits. Most deposits were brittle and opaque in character, stained with murexide, and all yielded x-ray diffraction patterns consistent with that of apatite (Fig. 2). There were no lines which could be matched either to other calcium phosphates, such as brushite or whitlockite, or to calcium carbonate (calcite). Sharp lines, as would be expected of samples with enamel contamination of the deposits, were never observed. While similarly brittle and stained deposits were noted on mandibular molars, they were relatively sparse. They were not collected for x-ray diffraction analysis because of fear of contamination of such samples with carious enamel.

Coronal fissure, approximal, and buccal smooth-surface carious lesions of mandibular teeth occurred, but no root surface lesions were noted (Fig. 3).

Bacteriological data.—Fig. 4 indicates that there were higher percentage recoveries of *S. sobrinus* 6715-13WT than of *A. viscosus* from rat teeth at 22 and 42 days post-inoculation. The percentage recoveries of 6715-13WT were significantly higher with RC-16-5 at 14 than with the other diets (p < 0.01 vs. RC-16-25; p < 0.005 vs. RC-16-50). *S. sobrinus* 6715-13WT was generally recovered at higher percentages at the second two sampling dates than the first, but the between-date values for the diet groups at days 22 and 42 were not significantly different from one another. *A. viscosus* recoveries were not assessed at day 14. The *A. viscosus* percentage recoveries were lower from animals consuming RC-16-50 at the 42-day recovery date, by comparison with RC-16-25 (p < 0.005) and with RC-16-5 (p < 0.001). Also, in this respect the results with RC-16-5 were different from those with RC-16-25 (p < 0.05) at this date. Between-date comparisons of the percentage recoveries of *A. viscosus* were different at day 22 and day 42 (p < 0.001) only for the group fed RC-16-50.

Calculus scores.—From 83 to 100% of calculus deposits

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Statistical analyses.—Groups were compared by one-way ANOVA and two-way ANOVA, with the least-significant-difference procedure used for isolating differences. Trends as a function of sucrose content of diets were also analyzed by regression analysis (Snedecor and Cochran, 1967; Sachs, 1982).

Bacteriological recoveries, expressed as percentages of the total recoverable flora, were arcsine-transformed to improve normality of distribution before parametric analysis. Deposit (calculus) scores and caries scores were analyzed without transformation of data. Data were plotted as means ± SEM.

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Fig. 2—X-ray diffraction patterns given by mineralized tooth deposits removed from maxillary molars of three rats and by a synthetic hydroxyapatite of poor crystallinity. (a) Synthetic apatite, (b) sample from a rat fed RC-16-5, (c) sample from a rat fed RC-16-25, and (d) sample from a rat fed RC-16-50 and pictured in Fig. 1. These patterns are typical of those from deposits in rats in each diet group.

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Fig. 3—Mandibular molar teeth of rat pictured in Fig. 1, illustrating (a) typical smooth-surface carious lesions of mandibular second and third molar buccal surfaces (arrows). Bar represents 1 mm. (b) After mandibular teeth were sliced in the occlusal-apical direction, typical sulcal and approximal lesions of mandibular molars are visible (arrows). Bar represents 1 mm.
were found on maxillary molar teeth (Fig. 5). The total scores (i.e., scores for maxillary plus mandibular molars) were significantly higher for RC-16-5 than for RC-16-50 groups at 23 days, but there was no other significant difference in scores as a function of the amount of sucrose in the diet. Scores at 43 days for each diet group were higher than those at 23 days (p < 0.05).

Caries scores.—Previous work has demonstrated that most carious lesions in rats occur in the mandibular dentition (Keyes, 1958a; Larson, 1981). By gross inspection of the rat dentitions, this appeared to be true of the present study also. Therefore, only mandibular lesions were scored. Total caries scores for mandibular teeth (Fig. 6) showed a clear trend to higher values as the amount of sucrose in the diet was increased (p < 0.0001). By ANOVA, scores for RC-16-5 and RC-16-25 groups were lower than for RC-16-50-fed rats at day 23 (p < 0.001 and p < 0.005, respectively) and at day 43 (p < 0.001 and p < 0.05, respectively). However, there was no statistically significant difference between total mandibular caries scores at the two dates for the groups fed RC-16-5 and RC-16-25.

Two-way ANOVA of values for individual tooth surfaces (Figs. 7 and 8) indicated that sulcal scores were higher for the RC-16-50 group than for the RC-16-25 and RC-16-5 groups (both, p < 0.001). The sulcal scores were different for the RC-16-25 and RC-16-5 groups as well (p < 0.05). Smooth-surface scores were higher for the RC-16-50 group than for the RC-16-25 group (p < 0.05) and the RC-16-5 group (p < 0.01), but there was no statistically significant difference between the scores for the RC-16-25 and RC-16-5 groups.

Discussion.

It is remarkable that plaques which formed on the maxillary molars of the rat, under the conditions of this study, mineralized conspicuously, yet the plaques which formed on the mandibular molars mineralized minimally and, by contrast, were associated with the development of carious lesions of those teeth.

During nearly 25 years of continuous experimental caries studies on SPFOM and specific pathogen-free Sprague-Dawley rats, our laboratory has never observed significant mineralization of either mandibular or maxillary plaque when other animal diets high in sucrose or glucose content were used, although those diets were relatively low in calcium and phosphate content. This experience is consistent with the literature (Tanzer, 1981). Conversely, high calcium and phosphate diets low in fermentable, simple carbohydrates have been reported to support calculus formation (Stewart and Burnett, 1960; Baer et al., 1961; Francis and Briner, 1969; Rolla et al., 1989).

The simultaneous development of calculus and substantial sulcal (fissure) and smooth-surface caries in these
rats is novel. While some mandibular tooth deposits clearly mineralized, as judged by their staining and brittle character, most apparently did not and were consumed by the dermestid beetles during the jaw-defleshing process. Rather, they became associated with tooth decalcification in a sucrose-associated, dose-response fashion, in patterns typical of caries in the rat (Keyes, 1956a; Larson, 1981).

Although it is not certain why maxillary tooth deposits led predominantly to calculus formation and mandibular ones led predominantly to caries in this rodent model, one may speculate that the proximity of at least the maxillary molar buccal surfaces to the opening of Stensen's ducts may have influenced the local pH, salivary environment, and tooth surface microflora to favor net plaque mineralization. However, it should be noted that the more distant maxillary molar palatal surfaces also evidenced such net mineralization. Others have reported that with high-calcium and -phosphorus diets used in experiments of much longer duration, maxillary molars of various rat strains developed more hard tooth surface deposits than did mandibular molars (Stewart and Burnett, 1960; Baer et al., 1961).

By contrast, the mandibular molar buccal surfaces, which are probably not more distant from the parotid duct orifices than the palatal surfaces of maxillary molars, evidenced net demineralization, as did the mandibular molar tooth fissures and approximal surfaces, and had minimal evidence of net plaque mineralization. Unfortunately, we know of no data pertaining to the flow of parotid and/or submandibular-sublingual or minor salivary gland secretions over the teeth and oral mucosa of the rat, analogous to those which are now appearing in the literature on human salivary films (Dawes et al., 1989).

Calculus-like deposits form on the teeth of germ-free rats (Fitzgerald and McDaniel, 1960) and mice (Baer and Newton, 1959). Biophysical studies have suggested these deposits to be apatitic (Glas and Krasse, 1962). Clearly, micro-organisms are not required for their formation. Nonetheless, abundant evidence supports participation of bacteria in this process in animals living in a microbially exposed world (ten Cate, 1989).

Calculus scores increased with the duration of the experimental trial, but were essentially independent of the amount of sucrose in the diet. Caries scores, however, increased not only with the duration of the experiment, but also with the amount of sucrose in the diet. A cautionary note is appropriate. The caries scores for the 5% sucrose-supplemented diet at day 23 may at first be regarded as surprisingly high. However, it must be recalled that early caries-scoring risks the identification of hypomineralized defects found in fissures of the rats' teeth which do not completely mineralize until the rats are much older (Francis and Briner, 1966). This is especially so when teeth have been stained with murexide, because the dye reacts with both incompletely mineralized tooth structure and with partially demineralized tooth structure reflective of caries. The caries scores in this study must be regarded as more reliable as the length of the study and the sucrose content of the diets increased.

Calculus scores were not clearly related to the recovery of S. sobrinus and A. viscosus from the dentition, consistent with other recent data (Tanzer et al., 1992). Carious lesions were far more abundant on the mandibular teeth, consistent with other experimental caries data for rats, and caries scores were more associated with high S. sobrinus recoveries than with A. viscosus recoveries (Tanzer, 1992). With a single exception, to our knowledge, others who have studied calculus formation in rats have failed to report simultaneous caries induction with sucrose, glucose, or corn starch diet contents as high as 66% by weight, using a variety of rat strains and basal diets and, in some cases, extremely long experimental periods (Stewart and Burnett, 1960; Baer et al., 1961; Rolla et al., 1989). The exception is the modicum of poorly mineralized tooth deposit formed on maxillary first molars which was termed calculus by Stewart and Burnett (1960) in rats fed cariogenic diet 580.

Because of the reported alkaline pH values for the saliva of the rat (as well as for the hamster and the macaque monkey) (Ericsson, 1964; Navia, 1977; Young and Schneyer, 1981), it could be tempting to speculate that calculus formed in the rat should be comprised mostly of calcium carbonate, calcite (Driessens and Verbeeck, 1989). However, all of the above-cited saliva pH data were gathered under states of para-sympathomimetic drug stimula-
tion or electrical stimulation of the visceromotor fibers to the submandibular and/or parotid glands. These data are inappropriate to be considered as reflecting the probable intra-oral pH or intraplaque pH of the rat (or of the hamster or macaque). Clearly, were alkaline pH values to exist during a major portion of the day in the rat's, macaque's, or hamster's mouth, development of caries would seem improbable. This is contrary to fact. Caries is a prominent feature of the pathobiology of these animals, as a large literature details (Tanzer, 1981). Furthermore, König et al. (1969) explicitly reported the number of minutes per day that an Osborne-Mendel rat eats ad libitum and the number of meals taken of a diet not greatly different from the one used in the present study. The rats ate, on average, 16.4 times daily for a total of about 200 minutes per 1440-minute day. Data also establish for the rat (Madison et al., 1991), monkey (Bowen, 1969), and hamster (Charlton et al., 1971) saliva and plaque pH to be mildly acidic to mildly alkaline in non parasymptomatically-stimulated animals. Clearly, the conditions of pH produced by intense parasympathetic nerve or drug stimulation have dubious relationship to pH conditions existing in the saliva and plaque of rats during about 86% of a day. Probably the most potent stimulus to salivation in the rat is the consumption of food, and most of the day the rat is not eating. It is in these circumstances that pH values of relevance to caries induction or calculus formation should be considered in attempts to explain the phenomena reported in the present study.

The present data demonstrate that, under the conditions of this study, detectable calcite was not formed. X-ray diffraction patterns of deposits on maxillary teeth, free of enamel contamination, corresponded with those of apatite of poor crystallinity and were typical of those found in bone, cementum, and calculus. Were calcite, brushite, or whitlockite present at more than 5% of the mineral content of the calculus samples studied, their presence would have been detected in the x-ray diffraction patterns. It cannot similarly be asserted that octacalcium phosphate was not present at less than 5% content in the calculus specimens, however, because its pattern is close to that of apatite (Smith, 1967; Eanes, 1973).

The present paper thus shows that it is possible for significant calculus formation and caries to be induced simultaneously in SPFOM rats fed a diet conducive to calculus formation and supplemented with sucrose. Deposits on teeth were found mostly on the maxillary dentition and were mostly brittle and opaque. Their staining and x-ray diffraction characteristics were consistent with apatitic calculus. Carious lesions mostly affected mandibular molars and were typical of those extensively described in the rat caries literature. It is now clearly possible to test topical agents, such as dentifrices or mouthrinses, designed to inhibit both calculus and caries induction in this SPFOM rat model.

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