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WARNER LAMBERT

January 24, 200

Director

The purpose of this document is to provide feedback to the Agency on three issues that were raises during the conference call of November 28, 2000 during which representatives of Warner-Lambert and FDA discussed clinical and statistical issues related to a proposed clinical study for Listerine with Fluoride.

The first two responses below are provided as follow-up to Agency comments originally provided by the Agency on July 21, 2000 and subsequently discussed on the November 28, 2000 teleconference.

I. Under the **Intra-orai Appliance Model:**

The Agency requested that the Sponsor provide a value for the difference of percent surface micro-hardness (SMH) recovery between positive and negative controls as a study validation criterion.

Following a review of the literature and discussion with outside experts, Sponsor proposes an absolute difference of 10% or greater of SMH recovery between the positive control and negative control. For example, if the negative control rinse exhibits a 10% recovery in SMH, it is reasonable to expect the positive control rinse to exhibit a recovery in SMH of 20% or greater.

The suggestion of a 10% difference of SMH recovery between the positive and negative control rinses is based primarily on experience of Dr. Domenick Zero, using dentifrices, in the intra-oral caries test. His review article, "*In situ* Caries Models" (Advances in Dental Research: 9 (3):2 14-230, 1995), a copy of which is in Appendix A, provides examples of data from four dentifrice studies in support of the Sponsor's recommendation. Based on the results for the gauze-covered ICT chips (Table I), it is reasonable to expect an absolute difference ≥ 10% between the positive and negative controls.

Table I. Difference in Mean % SMH Recovery
I 100 ppm Dentifrice versus Negative Control

Study Number	Data* Extracted From	Difference in Mean % SMH Recovery
I	Fig. IOB	12%
II	Fig. 10A	13%
III	Fig. 6, Fig. 10A	22%
IV	Fig. IOB, Fig. I I B	20%

* data from gauze covered remineralization model (Zero, 1995)

The above data were generated using procedures similar to those that Sponsor proposes to use for the ICT study.

II. Under the **Experimental Gingivitis Model:**

The Agency requested that the Sponsor propose a percent difference between positive and negative controls to serve as a criterion for study validation, and provide data to support the proposed difference.

As a result of the Agency's request, the Sponsor reviewed the study validation section in clinical study protocol 93 I - 1309 section 9.1.3, page 9, submitted May 15, 2000, in the context of plaque and gingival index results from eleven studies which compared Listerine antiseptic mouthrinse to a negative control. These studies all used the experimental gingivitis model accepted for final

formulation testing by the Plaque Products Subcommittee. Based on our discussion with the Agency and subsequent reassessment of the data from these studies, we have modified our original proposal.

The plaque and gingival index results from eleven 2-week studies comparing Listerine mouthrinse to a negative control are summarized in Table II. The plaque reductions seen in these studies are representative of those seen in the 6-month efficacy trials which were reviewed by the Plaque Products Subcommittee. The gingivitis reductions seen in two-week studies are generally lower than those seen in longer-term studies; this is consistent with published clinical findings which indicate that gingivitis usually resolves over a longer period. It should be recalled that the rationale for the inclusion of a clinical study for final formulation testing of essential oil-containing mouthrinses was based on the need to confirm the activity of new formulations against plaque biofilms *in situ*. Moreover, the long-term efficacy trials for the essential oil-containing mouthrinse standard consistently demonstrated a positive correlation between plaque reduction and gingivitis reduction. As a result, the Sponsor proposes that the criterion for validation of the experimental gingivitis study be based on plaque reduction alone. Based on the results for the two-week studies, it is reasonable to expect the positive control to reduce plaque by $\geq$ 15% compared to the negative control. Accordingly, the criterion proposed for study validation is that the standard essential oil mouthrinse formulation (positive control) produce $\geq$ 15% reduction in plaque as compared to the negative control at the 2-week examination period.

Table II. Percentage Reduction* at Two Weeks

	N	Mean	S.D.	Minimum	25"' Percentile	Median	75"' Percentile	Maximum
Plaque	11	23.6	4.9	16.0	19.6	23.3	28.0	32.7
Gingivitis	11	9.4	6.0	3.7	4.6	8.1	11.7	24.7

* Reductions, relative to negative control, in ascending order for eleven studies:
Plaque: 16.0, 17.7, 19.6, 21.9, 23.1, 23.3, 23.9, 25.2, 28.0, 28.0, 32.7
Gingivitis: 3.7, 4.3, 4.6, 4.9, 7.9, 8.1, 10.1, 10.2, 11.7, 13.6, 24.7

III. Regarding a clarification of the **Randomization Schedule:**

The ICT study utilizes a three by three crossover design. Three treatments (e.g., A, B and C) will be administered in 3 periods. Six treatment sequences (ABC, ACB, BAC, BCA, CAB, CBA) are planned, and each sequence will be randomly assigned to an equal number of subjects. Each subject will receive the three treatments following the sequence that is assigned. The timetable for visits and procedures is shown in Appendix A of each of the protocols.

Appendix A

Reprinted from *Advances in Dental Research*, Vol. 9, No. 3, 1995

In Situ Caries Models

D.T. Zero

Reprinted from *Advance-s in Dental Research,* Vol. **9,** No. 3, 1995

IN SITU CARIES MODELS

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Adv Dent Res 9(3):214-230, November, 1995

Abstract-By using *in situ* models, we have the potential to study both fundamental aspects of the caries process as well as more applied research problems such as the effect of food on dental caries and the role of fluoride in caries prevention in human subjects without actually causing caries in the **natural** dentition. The key experimental parameters that need to be considered in the development of an *in situ* model are the characteristics of the subject panel, the physical design of the model, the type of hard tissue substrate and the method of assessing mineral status, and the study design and clinical protocol. Each parameter must be carefully considered in relation to the objectives of the research, study design requirements, ethical considerations, impact on clinical relevance, and impact on the control of variation. The major source of variation associated with *in situ* models should be of biological and not experimental origin. The design and conduct of proper *in situ* model studies require a clear understanding of the caries process, sound analytical support, and a knowledge of how to work with research subjects to achieve a high level of **compliance**. Given the complex nature of caries, a combination of hard tissue **substrates**—including sound, surface-softened lesions and subsurface lesions-may be necessary to model all aspects of caries progression and prevention successfully. Internal validation of *in situ* models using fluoride dose-response controls is considered to be necessary for studies evaluating the efficacy of new fluoride dentifrice formulations.

Key **words:** Dental caries, dentifrices, fluorides. tooth **demineralization,** tooth **remineralization**.

Presented at the Conference on Clinical Aspects of De/Remineralization of Teeth, June I 1 - 14. 1994, Woodcliff Conference Center, Rochester, NY

In *situ* models have received increasing recognition as tools for the study of both fundamental and applied aspects of dental caries. The pioneering work of Koulourides and **Volker** (I 964) has blossomed into *the* development of various models which are now used all over the world in a wide range of applications. *In situ* models have been applied to the study of the mechanism of action of fluoride (Koulourides *et* al., 1974; Koulourides and Housch. **1983),** different fluoride dentifrice formulations (Schafer, 1989; Mellberg *et al.,* 1991; Stephen *et* al., 1992) and fluoride delivery systems (Corpron *et al.,* **1986a,b;** O'Reilly and Featherstone, **1987),** other remineralizing agents (Featherstone *et al.,* 1982; Pearce and Nelson, **1988),** microbial virulence factors (Zero *et* al., **1986a,b; Macpherson** *et* al., **1990),** the cariogenicity of foods (Brudevold *et al..* 1988; Kashket *et* al., 1988; Wefel and Jensen, 1992) as well as the protective effects of milk products (Silva *et al., 1986:* Reynolds, 1987; Featherstone and Zero, 1992). and the ability of gum chewing to modify the caries process (Kashket *et* al., 1989; Leach et al., 1989; Creanor *et al.,* 1992; Lamb *et* al.. 1993).

Recent interest in *in* situ models has been sparked by the Council on Dental Therapeutics of the American **Dental** Association, which decided in 1989 to accept a new. modified fluoride dentifrice based primarily on data from *in situ* models **(Naleway,** 1992). This led to two conferences in 1990 directed at *in situ* models and their role in predicting the efficacy of anti-caries agents. An outcome of these conferences was the realization that the various models needed to be validated based on their ability to show a dose response to different levels of fluoride.

In situ models have been challenged and scrutinized in a manner in which the other methods of caries assessment have not. I consider this to be a very positive and healthy development. Having *in situ* models run the fluoride **dose**-response gauntlet can only lead to their overall **improvement**. While this approach to model validation is not **beyond** criticism, it represents the only solid link that we have to clinical caries.

For the benefit of those who may be new to this **particular** research arena, I wish to start with a genera1 definition and a review of the advantages and disadvantages of *in situ* models.

DEFINITION

In situ caries models involve the use of **appliances** or other devices which create defined conditions in the **human** mouth that simulate the process of dental caries. **Ideally**, *in situ* models should serve as bridges between. **the** natural uncontrolled clinical situation and the highly **controlled** laboratory situation. Given the multi-factorial **nature** of dental caries, these models should include: a tooth **substrate**.

either enamel or dentin; the formation or presence of dental plaque with cariogenic potential; a carbohydrate challenge, either experimentally controlled or provided by the subject's normal diet; and time, determined by the length of the experimental period.

Studies involving in *siru* caries models are different from epidemiological studies **and** clinical trials in that they use relatively small panels of subjects. The intent of these model systems is to mimic what occurs in the natural caries process, yet provide clinically relevant information in a relatively short period of time without causing irreversible tissue changes in the natural dentition. Clinical caries trials require years to establish significant outcomes for therapeutic intervention, while intra-oral models may require as little as several months to provide results.

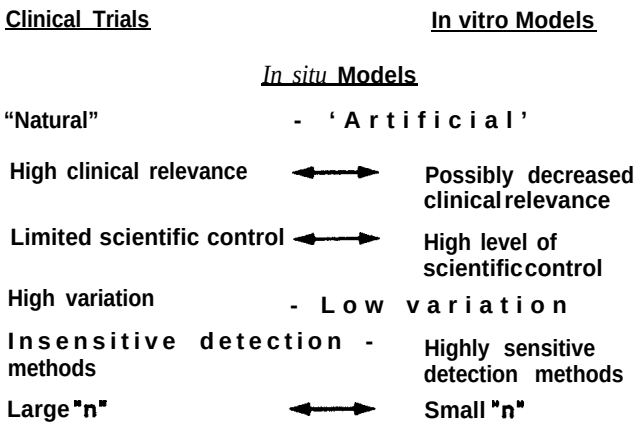

Fig. 1—Illustration of how in situ *models can be placed on the continuum between clinical trials and* in vitro *models.*

ADVANTAGES

The major advantages of in situ models are: (1) Studies are performed in the human mouth in contrast to *in virro* laboratory models or animal experimentation. *In vitro* studies have greatly improved our understanding of the caries process and the possible mechanisms by which fluoride has its anti-caries effect. However, the *in vivo* situation is complicated by dietary eating habits, the presence of physiologically secreted saliva, plaque of varying composition and thickness, and a pellicle-coated tooth surface (Bowen, 1983; Kleinberg *et al.*, 1983; Moreno and Zahradnik, 1979). These factors **and** possible interactions of therapeutic agents with the environment found in plaque and saliva are accounted for in the *in situ* model (Manning and Edgar, 1992; ten Cate and Marsh, 1994). Animal models also continue to serve a useful role in caries research (Stookey *et al.*, 1995). However, concerns regarding their direct application to humans have been raised because of differences in tooth morphology and composition, endogenous microflora, salivary composition, eating patterns, and food retention. (2) *In situ* studies facilitate control of experimental variables and a flexibility of experimental design in ways impossible to achieve with clinical trials. (3) These models facilitate the integration of various basic science analytical techniques, thus increasing the sensitivity and scientific validity of the methodology. Clinical studies are generally limited to insensitive clinical probing with a dental explorer and radiographs to identify dental caries at a relatively late stage of the disease process, when restorative intervention is the only alternative. (4) The short-term nature of the studies overcomes many of the ethical problems associated with studies involving human subjects. (5) There is generally a favorable cost factor compared with long-term clinical trials.

DISADVANTAGES

(1) Due to the nature of *in situ* studies, the number of subjects which can be involved is generally limited to between five and 40. Questions can be raised regarding how representative of the general population such a relatively small study population can be. (2) These studies are generally heavily dependent on compliance by the test subjects. Lack of compliance by a subject can have a major effect on the experimental outcome. (3) The conduct of high-quality *in siru* studies is very demanding, requiring both clinical and analytical expertise.

DIFFERENT TYPES OF *IN SITU* MODELS

It is very important to realize that under the heading of "*in siru* models" there is a **wide** range of models that are radically different in their responses to experimental variables. Wefel (1990) grouped *in situ* model systems into three categories: removable appliances, single-section models, and banding models. Manning and Edgar (1992) grouped them as *in vivo* models and *in situ* models for de- and remineraiization studies and also subdivided them into different methods. The *in vivo* models involve the **use** of natural dental tissues as the experimental substrate, while the *in situ* **models** use pieces of extracted teeth as the hard tissue substrate.

There are differences in the philosophical approaches that can be taken in *in situ* model development. A good starting place is the working hypothesis proposed by ten Cate (1992), which I support but with the addition of a caveat (in bold): "the predictive value of an in situ model with respect to a caries-preventive therapy is greatest when the **model** takes into account as many of the natural oral conditions as possible" **yet maintains control of variation such that scientifically valid outcomes can be detected with a practical number of subjects.**

As illustrated in Fig. 1, each *in situ* model can be placed at some point on the continuum between a clinical trial and an *in vitro* model study with regard to several parameters: degree of naturalness, clinical significance. scientific control, variation, sensitivity of the detection methods, and size of the sample.

It is not possible to have a completely "natural" model

without having the same degree of variation that occurs in *clinical trials. In situ* studies are limited regarding the number of subjects that can be tested. The constraints primarily involve the logistics of dealing with subjects and the labor-intensive and demanding analytical techniques required to support these studies. Therefore, the goal of *in siru* model development should be to control the variation in the model with the least impact on the clinical relevance.

I will not attempt to review each of the different types of *in situ* models. Excellent reviews on this subject have been previously published (Wefel, 1990; Manning and Edgar, 1992). What follows is a comprehensive review of the different factors that influence the behavior of *in situ* models. My intent is not to take a position on which model is better than another, but to illustrate by example how these different experimental parameters can modify the sensitivity and reliability of *in situ caries* models. The examples from my work will be referred to as Studies I-IV, which were a series of fluoride dose-response model validation studies involving modifications of the Koulourides partial denture model (Koulourides *et al.,* 1974).

EXPERIMENTAL PARAMETERS

The many experimental parameters involved in the design of *in situ* studies will be discussed under four major headings: (1) characteristics of the subject panel; (2) physical design of the model; (3) type of hard tissue substrate and method of assessing mineral status; and (4) study design and clinical protocol. Each of these parameters must be carefully considered in relation to the objectives of the research, the study design requirements, ethical considerations, impact on clinical relevance, and impact on the control of variation.

CHARACTERISTICS OF THE SUBJECT PANEL

In addressing this topic, I will be building on the previous reviews by ten Cate (1992) and Stookey (1992), which were published as part of the proceedings of the 1990 Consensus Conference. It has been suggested by ten Cate (1992) that the selection of subjects should be driven by the objectives of the *in situ* study. If the research objective is the determination of oral physiological parameters, then a subject panel should be selected that is representative of the population and does not favor or exclude selection of subgroups, unless of course the objective is to study particular subgroups such as drug-induced xerostomics. He recommended that subjects be selected based on specific characteristics, caries activity, saliva composition and flow patterns, and aspects of general health. If the research objective is to predict the efficacy of caries-preventive treatments, the conclusions based on an *in siru* subject panel should match the findings of a corresponding clinical trial. This approach may prove to be problematic, because the composition of the subject panel is only one of many factors which influence the response of *in siru* models. Altering the composition of the subject panel to achieve a desired experimental result may prove to be misleading and ultimately futile if the model is flawed in some other way.

From the perspective of clinical relevance. the study panel should be representative of the population for which the caries-preventive treatment is intended. For fluoride dentifrice studies, this covers almost the entire human life span. However, with the exception of the orthodontic banding model (Ogaard and Rølla, 1992), most models are limited to adult populations, due either to physical requirements of the model or to practical considerations. Many *in situ* models require that subjects have or need a dental prosthesis. Stookey (1992) recommended that adults rather than children are more appropriate as panelists for *in siru* studies when one considers that caries rate and response to fluoride treatment are similar for adults and children. Furthermore, adults are more likely to comply with clinical protocols and generally have greater availability for appointments.

Ethical considerations must also be taken into account in subject selection. Members of a study population must be capable of giving their informed consent before and during their participation in a clinical research study. The appropriateness of using "convenience populations" such as dental students and laboratory personnel for *in siru* studies must be carefully evaluated. In addition to ethical considerations, there is also the possibility of the introduction of experimental bias when studies involve individuals with special backgrounds and training in dentistry.

Another important factor is the impact of the subject panel on the variation in the response of the *in situ* model. Stookey *et al.* (1992) observed that there was a high level of variation among subjects in their model. One approach to subject selection is to include individuals with as wide a range of biological responses as possible. As discussed above, this may be appropriate if the objective is to study the effect of an oral physiological parameter. However, if the objective is to evaluate the effectiveness of a preventive treatment, this approach may compromise the ability of the *in situ* model to discriminate between control and experimental treatments. For practical reasons, *in situ* model studies are limited to an "n" of at most 40 subjects. If the inter-subject variation is a true reflection of the breadth of the general population, then the number of subjects necessary to have sufficient statistical power may be well beyond the maximum number of subjects that can be practically included in studies involving *in situ* models.

While I am riot advocating strict control of the types of subjects who participate in *in situ* studies, I recommend that the subject panels be standardized with regard to several parameters which are known to influence the caries process, such as medical health status. background fluoride exposure, and salivary flow rate. The standardization of subjects for *in siru* studies may not receive universal acceptance, but most investigators will agree that subjects should be well-characterized to permit proper interpretation of the data generated. The following is a list of factors which should bc considered in subject selection:

(1) *Demographics*—age, gender, and racial/ethnic

background. There are no clear scientific reasons to anticipate that differences in gender or racial background will influence the outcome of *in* situ studies. Where appropriate, the subject **panel for** in situ studies should have a balanced gender distribution and appropriate minority representation. It is noteworthy that for Federally funded research involving human subjects in the US, it is required to include minorities and both genders unless a clear, compelling reason for exclusion can be provided. This may be problematic in **some** areas of **the** US where minorities are underrepresented, as well as in countries that are more racially and ethnically homogeneous.

Cultural differences may involve different oral hygiene and dietary practices that could influence the response of an in *situ* model. This raises an interesting question regarding the universality of conclusions reached on the basis of *in situ* studies or even clinical trials conducted in different parts of the world.

(2) Medical health status-unless the objective of the *in situ* study is to evaluate a particular health-related parameter-for example, the effect of renal failure on dental caries-panelists should be in good general health. They should not have infectious diseases that increase the risk of cross-contamination of other subjects or the investigators. They should not have taken antibiotics two months before the study or during the study, since antibiotics will alter the oral flora and thus the response of the model. They should not be taking medications that **may** affect salivary flow.

(3) Dental health status-Susceptibility to the disease under study is an apparent requirement for study participants. Fifty years ago, before the widespread availability of fluoride, nearly the entire population of developed countries was susceptible to dental caries. Today, a high percentage of children in developed countries is caries-free. It is safe to assume that in the absence of fluoride this situation would rapidly reverse. While there may be subtle genetic differences that may increase or decrease caries susceptibility, it can be conjectured that dental caries can occur in the mouth of any individual, given the right set of conditions.

Dental caries is a highly localized and complex process which can occur on the surface of one tooth and not on the surface of the adjacent tooth. The design of an *in situ* model and the environment created by the model will have an overriding impact on the (demineralization/remineralization) response of a model. It is not necessary to select subjects who are highly caries-active, since it is possible to produce rapid demineralization of the test hard tissue substrate experimentally in the mouth of a subject whose natural dentition does not show any evidence of caries activity. It is desirable, however, to select subjects with a range of past caries experiences, to maximize the relevance of in *situ* studies. Many in *situ* models have the requirement that teeth be missing; therefore, the panelists tend to be individuals with moderate to high past caries experience. Subjects who are caries-active with unrestored carious lesions should be excluded from in *situ* studies, especially in studies that involve prolonged periods of placebo treatments.

For in *situ* models that require colonization by the subject's endogenous microflora, it is advisable that subjects have a minimum number of natural teeth. The microflora of fully edentulous individuals may have a cariogenic response different from that of dentate individuals (ten Cate et *al.,* 1992). We have adopted the criterion that subjects have at least eight natural teeth (Featherstone and Zero, 1992).

Potential subjects should also be screened for their periodontal health status. The placement of many types of in *situ* model devices on or adjacent to teeth may predispose the teeth to plaque accumulation and resultant gingival recession and tooth sensitivity. We have observed this situation using a "boat" appliance bonded to natural teeth (Featherstone and Zero, 1992). Another consideration is that individuals with gingival recession have altered oral clearance patterns compared with individuals without gingival recession and tend to retain higher concentrations of a test treatment such as fluoride in their mouths (Zero et *al.,* 1988). Thus, the inclusion of individuals with gingival recession may increase the inter-subject variation **of** an *in situ* study. Subjects with abnormal oral anatomy of congenital origin, or secondary to trauma or surgery, may also have altered oral distribution and clearance patterns and should generally not be included in subject panels.

The presence of dental restorations and appliances may also alter oral clearance patterns and may result in prolonged retention of food material and anti-caries agents such as fluoride. However, for most *in situ* model systems, this cannot be avoided. It is appropriate for ethical reasons that all restorations be in a good state of repair before the initiation of experimentation that could increase the risk of caries.

(4) Background fluoride exposure-Given the well-established anti-caries efficacy of fluoride in the water supply, it is essential that panelists be standardized regarding fluoride level in their water supply. Subjects should not be taking fluoride supplements for medical reasons. Furthermore, potential subjects should not have received professionally applied high-concentration fluoride treatments within two weeks before the start of an in *situ* study, because of the possibility of carry-over effects.

(5) Behavioral factors-Factors such as oral hygiene practices, daily activities and sleep habits, overall attitude. and ability to follow instructions will influence compliance with the study requirements. While no firm guidelines can be recommended, behavioral factors should be taken into consideration in the design of the study and at the time of subject selection.

(6) Dietary habits-These include the types of food\ and beverages, frequency and time of consumption, and eating sequence. Dietary habits can have a marked impact on the response of an *in situ* model. For most *in situ* models, the subject's diet is the main source of fermentable carbohydrates and resulting demineralization challenge to the model. The relationship of diet and dental caries (Bowen and Birkhed, 1986) as well as the interaction between diet and plaque micro-organisms are well-established (Zero, 1993).

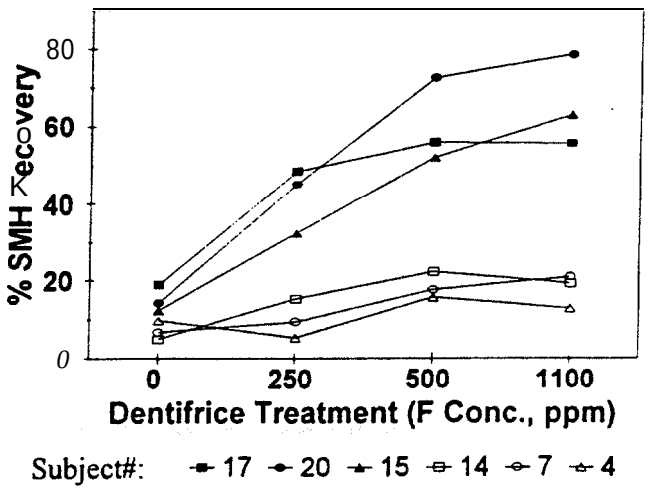

*Fig. 2—**Remineralization** responses of six subjects who participated in an in* situ *model validation study (Study IV), involving treatment with **dentifrice** containing either 0, 250, 500, or 1100 ppm **fluoride** (F). The test period lasted for I4 days. Three of the subjects (solid symbols) demonstrated an enhanced remineralization, expressed as % surface microhardness (SMH) recovery, in response to F dentifrice treatments, while three other subjects (open symbols) exhibited a much reduced response to F dentifrice treatment.*

Certain dietary practices, such as daily use of hard sugar-containing candy, which are held adjacent to the test site of *an in situ* model can lead to the almost complete destruction of enamel samples. Unfortunately, many aberrant dietary practices can be detected only after a study has been initiated. For studies involving a cross-over design, the use of a diet diary during each test leg can identify subjects who have changed their dietary pattern.

(7) Salivary factors-This topic is covered in much greater detail by Edgar and Higham (1995). Panels should consist of individuals with a wide range of salivary flow rates to represent the population accurately and maximize clinical relevance. However, this may also increase the inter-subject variation in the response of the *in* situ model. Individuals with greatly reduced salivary flow are likely to have a much greater demineralization challenge in an *in situ* model than subjects with normal salivary flow. We have adopted the criteria that subjects must have an unstimulated whole saliva flow rate ≥ 0.2 mL/min and a stimulated whole saliva flow rate ≥ 1.0 mL/min. These criteria would not apply when the objective of the research is to study a hyposalivatory population (Meyerowitz et *al.,* 1991).

Stookey (1992) has recommended that consideration also be given to evaluating other salivary parameters-such as pH, buffering capacity, and calcium and phosphate concentrations-which may influence the demineralization/remineralization response of *in situ* models. This author did not provide any specific information on what values should be used for inclusion/exclusion criteria. Unless more definitive guidelines can be provided, I do not believe that these additional measurements can be adequately justified at this time.

(8) Microbiologicfactors-This topic is covered in greater detail by Marsh (1995). Stookey (1992) recommended that determining the presence of *Streptococcus **mutans*** and lactobacillus in the oral flora of panelists could be used as an indicator of current caries potential. However, this requirement may be overly restrictive and may not add greatly to the responsiveness and validity of an *in situ* caries model. As will be discussed later, the local environment created by the *in situ* model system is an important factor in establishing the demineralization/remineralization potential of the model. Oral bacteria are capable of rapid adaptation to environmental conditions (Zero. 1993) that are influenced by the physical design of *an in situ* model.

If the research objective is to study the effect of a therapeutic agent on specific plaque organisms in relationship to an anti-caries effect, then microbiological screening may be appropriate. It may also be necessary to establish that the target micro-organism(s) represent a significant proportion of the plaque associated with the test sites.

*(9) **Demineralization/remineralization*** response--Subjects vary greatly in their response in studies involving *in situ* models. As shown in Fig. 2, two subsets of subjects who participated in a recent model validation study (Zero *et al.,* 1994a; Study IV) can be readily identified. The remineralization model that was used in this study involved the use of surface-softened bovine enamel covered with Dacron gauze. The results are expressed as % surface microhardness recovery, which is an indication of the extent of remineralization. This model generally favors net remineralization at the end of the two-week test period, even for the placebo treatment (Fig. 2). The two subsets can be grouped as high responders or low responders to fluoride dentifrice treatment. It was not possible to establish whether the difference in response is due to a greater demineralization challenge in the low responders. if there are inherent differences in the remineralization capability of these subjects, or a combination of the two. It is likely that each subject will be unique in this regard.

The above findings raise an interesting question: Can the sensitivity of *in situ* models be improved by pre-qualifying subjects based on their ability to meet certain conditions'? This approach has been recommended for studies involving plaque pH measurements (Schachtele. 1986) and is now routinely applied in most studies of this nature. When applied to *in situ* models, this could involve selecting only subjects who are able to generate fluoride dose response during screening tests, thus increasing the probability that the model will meet the validation criteria recommended by Proskin *et al.* (1992). The counter-argument is that clinical relevance will be lost by pre-qualifying subjects, because individuals who do not obtain the same benefit from anti-caries agents such as fluoride will not be accounted for.

PHYSICAL DESIGN OF THE *IN* SITU MODEL

The design of an in situ model must account for the complexity of the caries process. Caries-prone sites in the mouth represent unique micro-environments, which are influenced by the multitude of factors that ultimately determine if caries will occur. The variability associated with expression of dental caries is clearly evident during consideration of the number of subjects required for clinical trials with adequate statistical power. *In situ* models do not have the luxury of numbers and must attempt to control variability if meaningful results are expected. The physical structure of the model, the test site location, and the method of plaque formation are parameters that can be used by the investigator to help control variation. These parameters create the environmental conditions for plaque formation, access and retention of dietary substrate, salivary access, and access by anti-caries treatments, which can greatly influence the demineralization/remineralization response of the model, as is the case in the natural caries process (Fig. 3). Each of these parameters will be discussed separately.

(1) *Physical structure of the model.* Intra-oral models come in all shapes and sizes, ranging from the classical partial-denture model described by Koulourides *et al.* (1974) to fairly sophisticated models such as the intra-oral single-section model developed by Wefel *et* al. (1987). These model systems can be either fixed (cemented or bonded in place) or removable. *In situ* model systems that are fixed in the mouth have the advantage that subject compliance with wearing the appliance is not a factor. Model systems that use the subjects' normally worn partial dentures ensure a level of certainty that the subjects will be compliant with the study requirements. Removable devices that are specially constructed for research purposes may present a compliance problem in that subjects will either intentionally or inadvertently not wear the appliance.

In addition to the basic design of the model, another important consideration is the reproducibility of the physical structure of the model between tests in the same subject (when using a cross-over design) and between different subjects. Many models rely on the use of dental materials to construct appliances or crowns that carry the hard tissue substrate. As discussed above, the physical structure of the model can have a major influence on several parameters that affect the response of a model (Fig. 3). In *situ* model systems which cannot standardize the physical structure of the model are likely to have inherently greater inter-subject and intra-subject variation.

(2) Test site location. The palatal area, lingual area, buccal fold area, and the teeth and edentulous spaces in different areas of the mouth have all been used as test sites for retaining hard tissue specimens (Manning and Edgar, 1992). The test site location will also greatly influence the response of an *in* situ model (Fig. 3). The clearance patterns of dietary substrate and anti-caries agents such as fluoride will vary in different locations of the mouth, depending on the rate of salivary flow, proximity to the major salivary gland duct

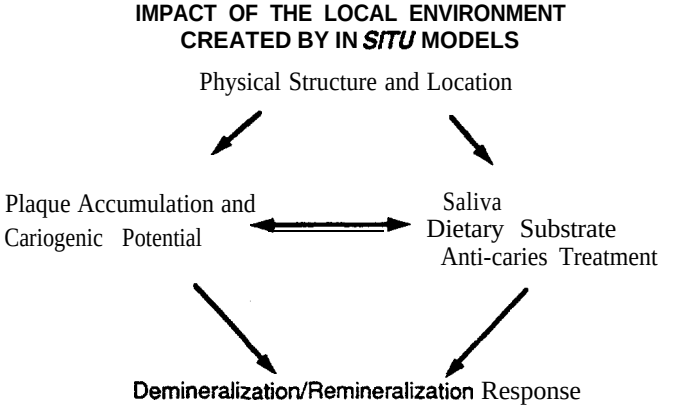

Fig. 3—Illustration of the complex relationship of the physical structure and intra-oral location of an in situ *model and their effects on different parameters that influence the response of the model.*

orifices, and anatomical factors (Weatherell *et al., 1986;* Watanabe, 1992; Zero *et al.,* 1992b). Furthermore, there are differences in salivary film thickness and salivary film velocity in different locations of the mouth that may influence the cariogenic potential at a specific site (Dawes. 1993). These parameters may also be modified by the presence of the.intra-oral device or appliance.

The location where the hard tissue substrate is placed in the mouth can be a considerable source of intra-subject and inter-subject variation. We have observed that, even in a well-controlled *in situ* model, differences in the response of test sites on the left and right sides of the mouth can occur within the same subject (Fig. 4). These data are a subset from the model validation study referred to earlier (Zero *et* al.. 1994a; Study IV). In each of the four subjects, a consistent pattern of response for the left and right test sites was evident across all treatments. For two of the subjects (Figs. 4a.b). there was consistently greater remineralization (% SMH recovery) observed at the left test site, while for the other two subjects (Figs. 4c,d), there was consistently greater remineralization observed at the right test site. This observation is consistent with the findings of Mellberg *et al* (1992). who, using the thin-section sandwich model, reported marked variation in the mineral changes between right and left sites.

Fig. 5 shows the fluoride dose response for one subject in two different models (demineralization model and remineralization model) that were run in parallel in Study IV The demineralization model involved the use of sound bovine enamel covered with Dacron gauze. The results are expressed as % surface microhardness change. which is an indication of the extent of enamel demineralization. The remineralization model involved the use of surface-softened bovine enamel covered with Dacron gauze. The results are expressed as % surface microhardness recovery, which is an indication of the extent of remineralization. The sound (demineralization

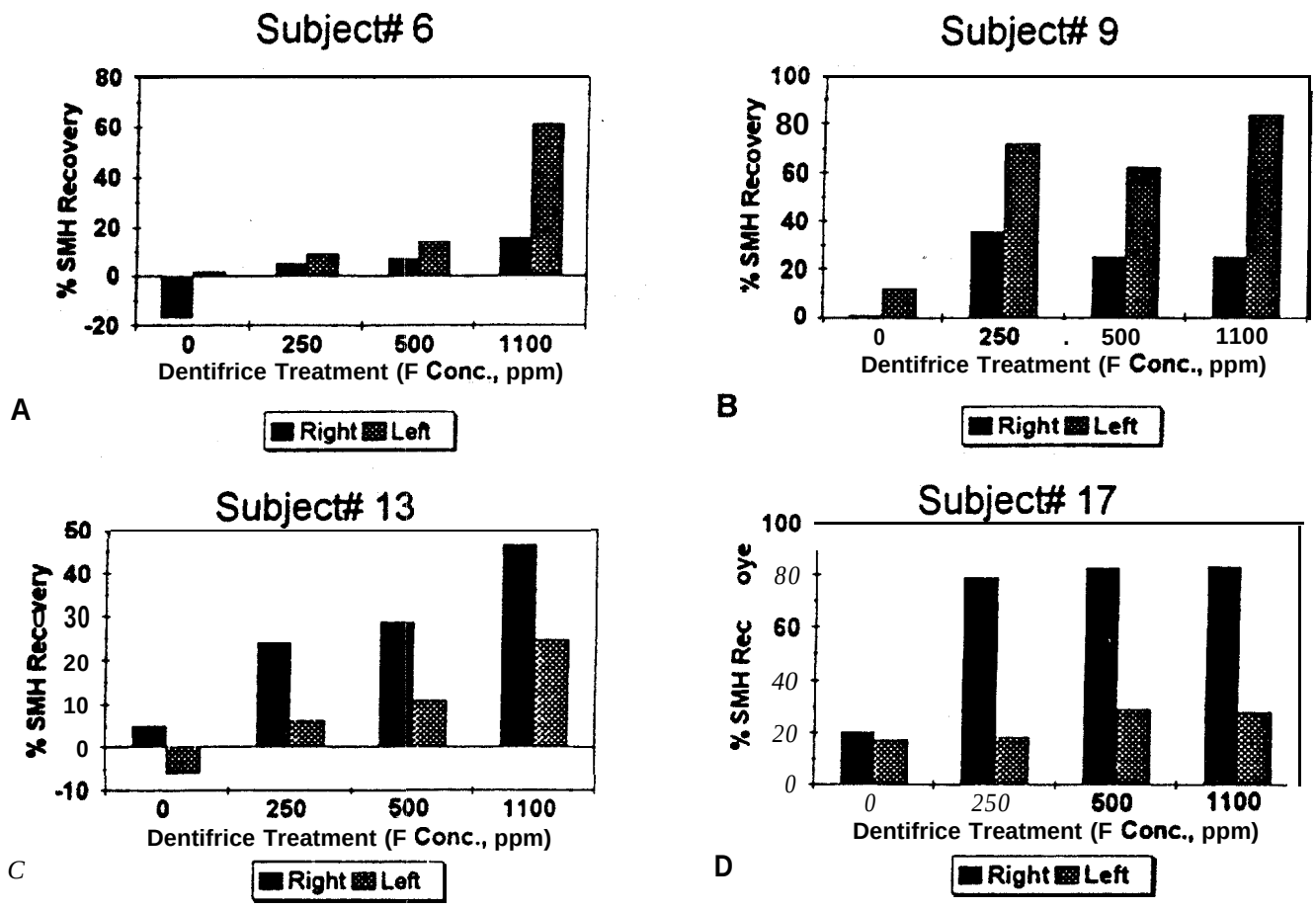

Fig. 4—Side-to-side differences in the responses of four subjects who participated in an in situ model validation study (Study IV) involving treatments with dentifrices containing either 0, 250, 500, or 1100 ppm fluoride (F). Two of the subjects exhibited greater remineralization, expressed as % surface microhardness (SMH) recovery, on the left side than on the right side (a and b), while for the other two subjects, the effect was greater on the right side (c and d).

model) and surface-softened (remineralization model) enamel blocks were mounted adjacent to each other in the buccal flange area of each subject's partial denture. It appears that the weaker remineralization response of the right side of the mouth compared with the left side was due to a stronger demineralization challenge on the right side. In this particular subject, this was most likely due to a chewing preference for the right side of the mouth. The findings from the other subjects in the study were not as easy to interpret, based on a similar comparison of the findings from the two models.

These findings support the contention that different test site locations in the mouth create unique environments that can influence the response of an *in situ* model. It can be anticipated that the difference in the response of test sites located in different regions of the mouth-for example, the maxillary anterior vs. the mandibular lingual-will be greater than observed in contralateral locations. The standardization of the intra-oral test location of an *in situ* model may help to reduce variation. Our group has elected to use the mandibular buccal flange area as the test site for all of our *in situ* studies with the Koulourides-style model.

(3) Method of plaque accumulation. The design of the *in situ* model and the local environment created at the test site will influence both the composition and thickness of the plaque that forms over the tooth test site. The composition of the plaque and its thickness will. in turn, markedly influence the behavior of the model with regard to both demineralization and remineralization as well as the ability of anti-caries treatments such as fluoride to reach the surface of the tooth specimen.

To illustrate the role of plaque composition and thickness. I will review data from several different types of models that are used in our laboratory. Fig. 6 shows data from a fluoride dose-response study using a partial-denture model (Study III). This study involved a simultaneous comparison of the response of two types of remineralization models, a gauze-covered model and a gauze-free model. The gauze-free model consistently resulted in a greater remineralization response than the gauze-covered model for all treatment conditions. Both models used surface-softened bovine enamel as the hard

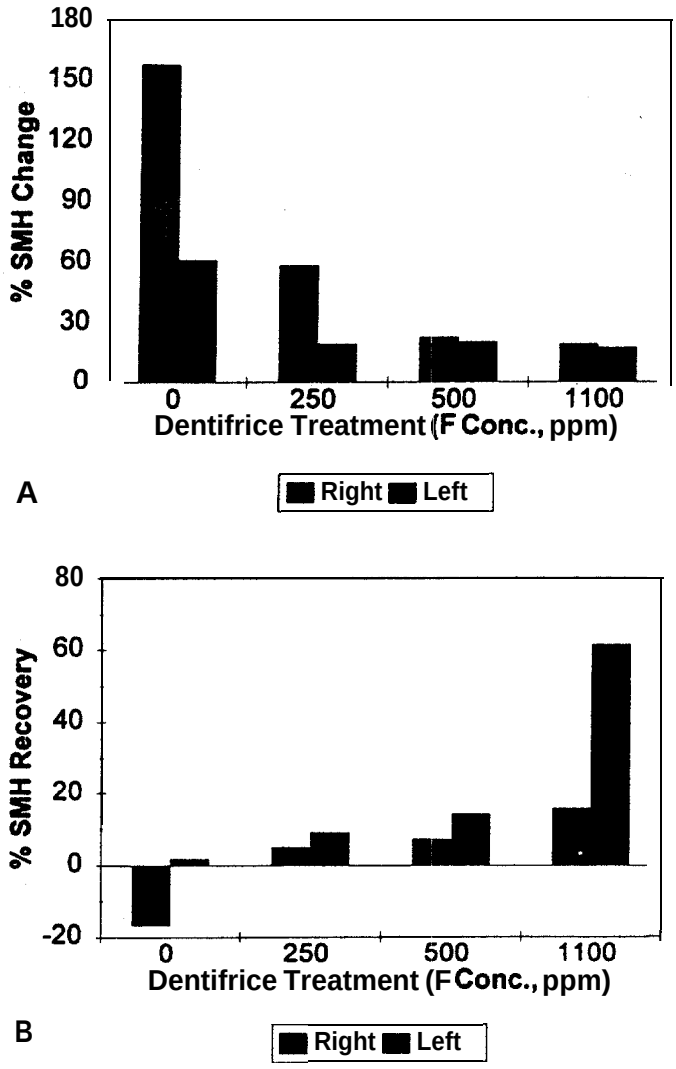

Fig. 5—Comparison of the side-to-side differences for one subject who participated in an in situ *model validation study (Study IV) involving treatments with dentifrices containing either 0, 250, 500, or 1100 ppm fluoride (F). (a) Demineralization model-the extent of enamel demineralization of sound bovine enamel, expressed as the % surface microhardness (SMH) change. (b) Remineralization model—the extent of enamel remineralization of surface-softened enamel, expressed as the % SMH recovery.*

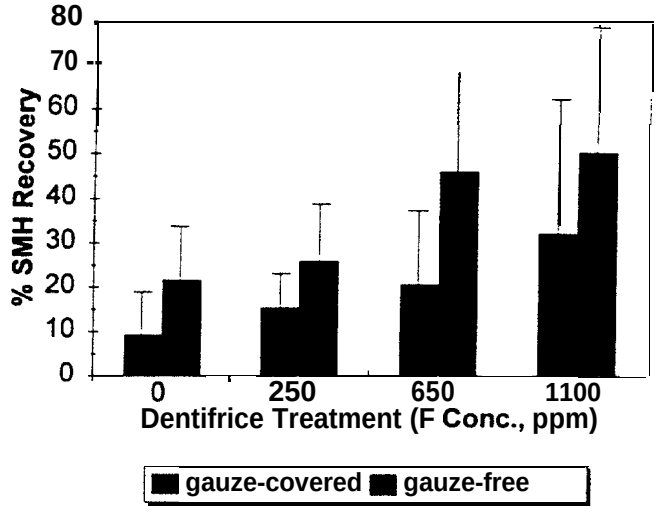

Fig. 6—Simultaneous comparison of the fluoride dose responses of the gauze-covered remineralization model and the gauze-free remineralization model (Study III). Data are reported as mean values and expressed as % surface microhardness (SMH) recovery (remineralization). The bar represents the standard deviation; n = 13 subjects.

tissue substrate mounted flush with the surface of the buccal flange of each subject's partial denture. It is important to realize that the enamel blocks used in these models were placed next to each other, and the only difference in the two models was the presence of gauze.

The gauze-free model is representative of exposed smooth tooth surfaces where plaque accumulation is more limited due to the abrasive force of the oral soft tissues and oral hygiene practices. Thus, there is less of a demineralization challenge, and the tooth surface is more accessible to the beneficial effects of saliva and anti-caries treatments such as fluoride.

For the gauze-covered model, the presence of the gauze encourages the accumulation of plaque and also alters the diffusion of ions to the enamel surface (Mellberg, 1992). This causes a greater acid challenge to the enamel surface by increasing the bacterial biomass and by altering the diffusion characteristics such that salivary constituents are restricted from reaching the deeper plaque layer adjacent to the enamel surface. This leads to a greater demineralization challenge to the enamel surface, and thus less net remineralization for the gauze-covered model. This model reproduces the condition that exists in caries-prone sites such as interproximal areas and pits and fissures, where ion diffusion tends to be restricted. In addition to the greater demineralization challenge, the presence of gauze may also restrict diffusion of anti-caries agents such as fluoride to the enamel surface (Mellberg, 1992). The combination of a greater demineralization challenge and restricted diffusion of fluoride may account for less net remineralization in the gauze-covered model than in the gauze-free model (Fig. 6).

Additional information on the importance of plaque thickness and plaque composition has been obtained by means of the intra-oral enamel demineralization test (IEDT) (Zero *et al.*, 1992a). This model is a modification of the model originally introduced by Brudevold *et al.* (1984). With this model, test plaque of different compositions can be evaluated under highly controlled conditions. *Streptococcus mutans* test plaque containing glucan was found to have far greater enamel demineralization potential at thicker plaque

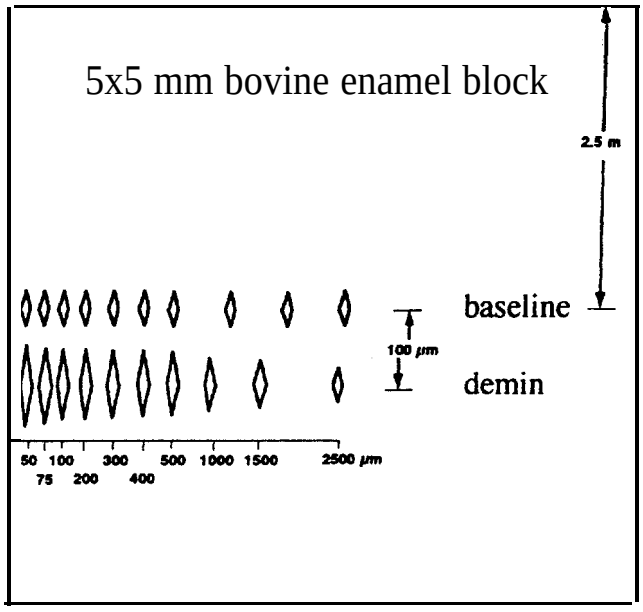

Fig. 7-Diagrammatic representation of the sites where indentations were made on the bovine enamel block to determine changes in surface microhardness before (baseline) and after (demin) the 45-minute test period. These indentation sites correspond to the effective plaque thickness at each site when the enamel blocks are loaded into the intra-oral enamel demineralization test model.

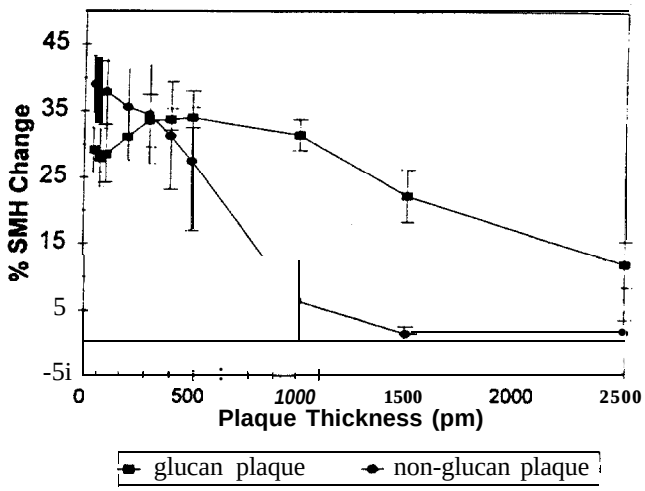

Fig. 8-Comparison of a giucan-containing plaque with non-glucan plaque in the intra-oral enamel demineralization test model (Zero et al., 1992a). This modtjied revision of the model simulates the demineralizing effect of different plaque thicknesses ranging from 50 to 2500 pm. The data are reported as mean % surface microhardness (SMH) change (demineralization). The bar represents the standard deviation; n = 5 subjects.

layers *than an S. mutans* test plaque without glucan (Zero *et al.,* 1992a). Consequently, the composition of plaque will affect its diffusion properties and cariogenic potential.

More recent work with a modified version of this model has further clarified the relationship between the demineralization potential of plaque and plaque thickness/composition. The modification involved expanding the number of measurement sites such that demineralization associated with thinner plaque layers could be assessed. The measurement sites corresponded to plaque thicknesses of 50, 75, 100,200, 300, 400, 500, 1000, 1500, and 2500 pm (Fig. 7). With this modified approach, new insight into the relationship between plaque thickness and plaque composition and enamel demineralization has emerged (Fig. 8). When a highly cellular plaque mass (non-glucan plaque) was challenged with a 20% sucrose rinse, the extent of demineralization was greatest at the enamel site covered with a 50-µm-thick plaque and decreased with increasing plaque thickness up to 1500 µm, where no detectable demineralization could be observed. For the glucan-containing plaque, the extent of demineralization initially increased as plaque thickness increased up to 500 µm, gradually decreased, yet still remained well above baseline at the 2500-µm site.

These findings definitively show how glucan modifies the diffusion properties of plaque. For a thin plaque layer (under 500 pm). the presence of glucan in plaque decreased the demineralization potential of plaque. This is most likely due to increased diffusion of saliva into the plaque mass, resulting in more rapid clearance of sucrose substrate and more effective acid neutralization by salivary buffers. At deeper plaque layers (greater than 500 µm), the diffusion of carbohydrate substrate resulting from the 20% sucrose rinse dominates the process, and thus the presence of glucan enhances demineralization compared with plaque without glucan.

There is every reason to believe that a similar relationship between plaque thickness and demineralization exists in naturally formed plaque. Based on computer modeling. Dawes and Dibdin (1986) predicted that a thinner plaque will have greater demineralization potential than a thicker plaque. The optimum thickness of plaque with the greatest demineralization potential is dependent on the concentration of sugar in saliva. These predictions fit very well with the IEDT model data, with the caveat that plaque composition also needs to be factored into the model.

The importance of controlling plaque thickness in *in situ* models cannot be overemphasized. The above findings indicate that differences in plaque thickness of 0.5 mm or less can have a profound effect on the response of an *in situ* model. This may be the one area where control of variation may have to be given a higher priority than the maintenance of strict clinical relevance by attempting to model natural plaque formation. For our partial-denture models, we have elected to use Dacron gauze to control plaque thickness. By placing the gauze directly on a flat enamel surface and mounting the enamel blocks flush with the buccal acrylic

flange of the partial denture, we limit plaque thickness by the thickness of the gauze. Other methods of encouraging plaque formation at *in situ* model test sites-such as recessing enamel blocks below the acrylic flange (Dijkman *et al.*, 1986; Corpron *et* al., 1986b) and mounting hard tissue specimens interproximally in hollows prepared in gold crowns (Wefel *et* al., 1987)—have the advantage that a more natural plaque may form. However, these models are likely to be more inconsistent, due to their inability to control plaque thickness at the test site. Models which attempt to simulate natural plaque formation must also attempt to standardize the conditions under which the plaque forms such that plaque thickness is carefully controlled. This can be very challenging in the modeling of an inter-proximal embrasure where the placement of the hard tissue specimen, the contours of the adjacent teeth or restorations, natural cleaning forces such as the tongue and cheek movement, and oral hygiene practices all influence the effective thickness of plaque at the test site.

TYPE OF HARD TISSUE SUBSTRATE AND METHODS OF ASSESSING MINERAL STATUS

An excellent review of the different types of tooth material used in *in situ* studies has been previously published by Mellberg (1992). My treatment of this topic will be limited to a brief overview, and then I will focus on work by our group on the initial enamel surface lesion. Wefel (1995) presents a more detailed review of the sub-surface lesion and the use of dentin as a test substrate.

It appears that for *every in situ* model, there is a different hard tissue substrate used with the model (see Manning and Edgar, 1992). Most model systems use either human or bovine tooth material. Both enamel and root surfaces have been evaluated in these models. The tooth material can be prepared either as blocks or as single sections. The surface of the material can be either natural or abraded and polished. Sound or partially demineralized tooth material can be utilized. Sound enamel can be used to measure the extent of net demineralization occurring in the model, while partially demineralized enamel can be used to measure the extent of both further demineralization as well as net remineralization in the model. Partial demineralization or incipient caries can be developed *in vitro* under well-controlled laboratory conditions as well as by use of the *in situ* model.

Human teeth must be considered the most appropriate source of hard tissue substrate for *in situ* studies from the perspective of clinical relevance. However, human teeth are of a highly variable composition, due to genetic influences, environmental conditions (diet, fluoride exposure, prior caries challenge), and age (Mellberg, 1993). These differences result in large variations in their response under *in vitro* acid challenge and *in situ* test conditions. Their relatively small size and curved surface do not permit them to be used in experiments requiring flat surfaces of uniform thickness, such as those required in certain *in situ* models (Zero *et al.*, 1992a).

Our laboratory has elected to use highly standardized bovine enamel blocks as our hard tissue substrate, and although this approach is not without criticism, it has a number of advantages. Bovine enamel is readily available, while human enamel is becoming increasingly more difficult to obtain. Bovine enamel has more uniform composition than human enamel, and thus provides a less variable response to both cariogenic challenge and anti-caries treatments (Mellberg, 1992). Although bovine enamel is more porous than human enamel and demineralizes faster (Featherstone and Mellberg, 1981; Edmunds *et al.*, 1988), these differences result in quantitative differences and not qualitative differences in behavior in *in situ* models (Mellberg, 1992). Overall, bovine enamel can be considered an acceptable alternative to human enamel and may offer advantages to human enamel for *in situ* studies by decreasing the response time and variability of the hard tissue substrate response in the model (Mellberg, 1992).

Since the proper sterilization of hard tissue substrates must now be considered mandatory for all *in situ* studies, consideration must also be given to whether a sterilization procedure alters the properties of hard tissue substrate. Chandler (1990) reported that sterilization with ethylene oxide or gamma irradiation did not alter the microhardness properties of enamel; however, autoclaving was found to decrease enamel microhardness significantly. Our group has not detected any change in the *in vitro* acid solubility of human or bovine enamel after ethylene oxide sterilization.

It is now widely recognized that the type of hard tissue substrate will have a major impact on the response of an *in situ* model, and that in the case of pre-formed lesions, different methods of preparing a lesion will alter its demineralization/remineralization response in the *in situ* model (Mellberg, 1992; ten Cate *et al.*, 1992). To complicate this matter further, there are 10 or more experimental techniques available for assessing demineralization or reminrralization (ten Bosch and Angmar-Månsson, 199 1: Arends and ten Bosch, 1992). There also appears to be a wide variation in the protocols used among different investigators using the same basic approach (White *et al.*, 1992).

Although this aspect of *in situ* model development has received more attention than any other, it remains unresolved as to which type of hard tissue substrate best represents the "natural" caries process and which type of substrate has the best predictive value of the clinical effectiveness of a caries-preventive treatment. These are clearly two different research objectives, and the ideal hard tissue substrate may not be the same for both. Given the ambiguity of this aspect of in *situ* model research, I believe that it is appropriate to revisit the theoretical considerations involved in the selection of a hard tissue substrate.

The choice of hard tissue substrate can be approached by two different working hypotheses: (1) that the net loss or gain of subsurface mineral is the main factor which determine\ if clinical dental caries will occur; and (2) that interactions with the tooth surface are the main factors that determine if clinical dental caries will occur.

Most caries research over the past 30 years had focused on the formation and repair of the subsurface lesion (first hypothesis). I wish to challenge the concept that the "subsurface lesion" is the only appropriate hard tissue substrate for use in *in situ* models. Furthermore, I believe that there is some truth in the statement that the use of subsurface lesions has been driven to a large extent by the requirements of the technology available to measure loss or gain of mineral and not necessarily by the clinical relevance of the subsurface lesion as the ideal hard tissue substrate for in situ studies.

While subsurface lesion (white spot) formation is clearly a reversible stage of the clinical caries process, it does not always precede cavity formation. Many early lesions have been described as surface softening that progresses to loss of surface material and eventually clinical caries. The white spot may be looked upon as a partially arrested lesion, which may or may not progress to a frank cavitation.

The use of subsurface lesions in in *situ* studies presents a number of challenges: (1) Subsurface lesions are very difficult to standardize, even under well-controlled in *vitro* conditions, and thus can be a source of considerable variation in in *situ* model response (Mellberg, 1992; ten Cate *et* al.. 1992). (2) The technique of transverse microradiography (TMR) is the most established method for the quantification of mineral loss or gain. However, this approach is technically very demanding and subject to wide variations in the conditions of analysis (White et *al.*, 1992). (3) The main interactions of hard tissue substrate with the oral environment occur at the surface layer of the sample. This is especially true for fluoride, which primarily interacts with the outermost surface layer (Iijima and Koulourides, 1989). Transverse microradiography (TMR) and other methods such as cross-sectional microhardness and polarized light are limited to the study of changes in the subsurface region and cannot accurately evaluate changes occurring in the outer 25 pm of surface enamel (Featherstone, 1992). (4) Remineralization of subsurface lesions with an intact surface layer occurs only to a limited extent and is more likely to be found with advanced subsurface lesions (Mellberg, 1992). The surface layer needs to remain porous to permit remineralization of the body of the lesion to occur. This can be achieved only by starting with a lesion with a porous surface layer and/or by creating a high-challenge caries environment in the in *situ* model to keep the surface layer porous. Establishing a consistent fluoride dose response of an in situ model that uses subsurface lesions as the hard tissue substrate may prove to be very challenging, because fluoride has a more limited anti-caries effect in high caries challenge sites (Øgaard and Rølla, 1992).

In support of the contention that interactions with the tooth surface are the main factors that determine if clinical caries will occur (second working hypothesis): (1) Early lesions do not have intact surface layers, and the first stage of caries formation involves surface-softening (Moreno and Zahradnik, 1974; Thylstrup et al., 1983; Arends and Christofferson, 1986). (2) The outer enamel surface is the tooth material that is in direct contact with dental plaque. The fluid phase of plaque (plaque fluid) is largely responsible for creating the conditions that favor either dernineralization or remineralization. (3) Surface lesions are more responsive to the oral environment and tend to remineralize very rapidly (Arends and Gelhard, 1983; Dijkman et *al.*, 1986). (4) Fluoride interacts primarily with the outer surface enamel with regard to uptake, inhibition of demineralization, and enhancement of remineralization. The early surface-softened lesion is the consequence of frequent intermittent exposure to plaque acids produced throughout the day. Fluoride is most likely having a major part of its effect by preventing demineralization from occurring and/or enhancing remineralization secondary to this intermittent demineralization. There is clinical evidence that fluoride is most effective in inhibiting the initiation of caries and much less effective in inhibiting lesion progression (Bjamason and Finnbogason, 1991). We recently found that the enhancement of remineralization of the very early surface lesion by fluoride dentifrice can be detected after only a four-hour period of intra-oral exposure (Zero *et* al., 19946).

While we do not have definitive scientific evidence to support either the first or second working hypothesis, evaluation of the early surface changes in enamel may prove to be the most sensitive measure of fluoride efficacy and may have the greatest predictive value with respect to anti-caries effectiveness. There are several methods for assessing changes occurring in the outermost surface layer of enamel (ten Bosch and Angmar-Mlnsson, 1991; Arends and ten Bosch, 1992). These include the iodide permeability test, the surface microhardness (SMH) test, and newer methods such as wavelength-independent microradiography. My discussion will be confined to the use of the SMH test.

The SMH test has been widely used to study enamel demineralization and remineralization occurring in in *situ* caries models (Koulourides et *al.*, 1974; Gelhard et al.. 1979; Corpron *et al.*, 1986a,b). These studies all used a 500-g load to study fairly advanced changes in the mineral status of enamel. More recently, the SMH test has been used with a reduced load (50 g). which has greatly increased the sensitivity of the method (Buskes *et al.*, 1987; Pearce and Nelson. 1988; Zero *et al.*, 1990). thus permitting changes in the outermost layer of enamel to be evaluated. This technique has been criticized based mainly on the conclusion reached by Arends and Gelhard (1983), that hardness increase is not identical to remineralization. This conclusion is largely bused on the concern that surface microhardness measuremcntr Jo not allow for localization of mineral loss or gain, or for the detection of redistribution of existing mineral within lesions following exposure in the oral environment. This position is reasonable if the objective of the research is to study in great detail the remineralization process in the mouth. On the other hand, if the intent is to study early changes in the enamel surface or to predict the outcome of an anti-caries treatment. this concern may not be warranted. White (1987) reported that for early carious lesions of shallow depth (between 25 and 50 μm), the net remineralization as measured by the

SMH was highly correlated with remineralization as measured by microradiography ($r^2 = 0.94$; $p < 0.01$).

My research has focused on the very early changes in surface enamel, using the SMH test as an indication of whether conditions in the oral environment favor demineralization or remineralization (the second working hypothesis). We have found the SMH test to be a highly sensitive and reproducible method for studying the very early stages of in *situ* enamel demineralization (Zero *et* al., 1992a, 1994a) and enamel remineralization (Zero et *al.*, 1994a,b).

One note of caution when using methods such as SMH that measure changes in the enamel surface as a predictive tool of clinical efficacy: It is possible that in *situ* models that evaluate enamel remineralization may show reduced effectiveness when testing fluoride formulations containing surface-active agents, such as pyrophosphate (Zero *et al.*, 1994b). There are some clinical data supporting the contention that anti-calculus agents may not interfere with the clinical anti-caries effectiveness of fluoride dentifrices (Lu *et al.*, 1985; Koch *et al.*, 1990). However, these studies cannot be considered conclusive, and this very important issue deserves further study from both a clinical and a modeling standpoint.

In summary, the choice of hard tissue substrate and method of assessing mineral status remain the most challenging areas of *in situ* development. Given the complex nature of the process of dental caries, it may require a combination of approaches to achieve the goal of having high predictive capability for all possible clinical outcomes. It may be necessary to model caries with multiple hard tissue substrates, including sound enamel and surface-softened and subsurface lesions, which could be combined in one in *situ* model system or in multiple studies using different models.

STUDY DESIGN AND CLINICAL PROTOCOL

There are many factors that need to be taken into consideration in the actual study design:

(1) The number of *subjects--The* appropriate number of subjects can range from five to as many as 40. depending upon the nature of the model, the variability of the model, and the research question. It is essential that the number of subjects included in *in situ* studies be determined based on statistical power calculations.

(2) Appropriate controls-Depending upon the research question, both positive and negative controls may be appropriate. In addition, when fluoride dentifrices are studied, dose-response controls should be included to validate the model internally.

(3) Cross-over vs. monadic design-The research question and the type of *in situ* model will determine which design is most appropriate. A cross-over design is preferred because of the relatively small size of subject panels and the fact that groups of subjects cannot be properly stratified based on all the parameters that affect the response of *in situ* models.

(4) Length of rest period-The length of the study period can range from as short as 45 minutes to as long as 6 months, depending upon the type of model, the research question, and the sensitivity of the analytical methods.

(5) Type of dietary challenge-Most models rely on the normal diet of the subjects to provide the dietary challenge to the model, We routinely have our subjects complete a five-day diet diary during each test leg to determine if there has been any major change in their diet that could alter the response of the model and invalidate the cross-over design. Other investigators have supplemented the normal diet with standardized snacks (Leach *et al.*, 1989; Creanor *et al.*, 1992). In models involving removable appliances, it is also possible to supplement the dietary challenge with an extra-oral sugar challenge by submerging the appliance in sucrose solutions (Koulourides *et al.*, 1974; Featherstone and Zero, 1992). However, this measure may increase the artificiality and variation of the model (see below, Fig. 10).

(6) The method andfrequency of delivery of a test agent—Either the normal practices of the test subject or a well-defined clinical protocol can be used, depending on the research objectives. We have adopted the practice of standardizing the delivery of test agents. Fluoride dentifrices are provided to the subjects in plastic syringes that deliver I .5 g of the test dentifrice. The subjects are instructed to brush for one min and then to expectorate and rinse with 15 mL of tap water.

(7) Standardized lead-in procedures-These may involve a dental prophylaxis and the use of placebo (non-fluoride) dentifrice several days before the start of each test leg. The intention of the lead-in procedure is to standardize the oral condition at the start of a test as much as possible to minimize variation and possible carry-over effects.

(8) The length of the wash-out period between test treatments-This is an important design consideration if a carry-over treatment effect is anticipated. We generally use a one-week wash-out period. Other investigators use two weeks or longer (Stephen *et al.*, 1992). A practical concern is the effect of the wash-out period on the overall length of studies involving multiple test treatments. The longer a study runs. the greater the possibility of behavioral changes in the subjects or loss of subjects due to attrition.

(9) The use of compliance indicators-These may include the sampling of salivary fluoride concentrations, check-off sheets, and product disappearance information. The importance of subject compliance cannot be overemphasized. The subjects' understanding of the clinical protocol must be monitored constantly. We provide written instructions to the subjects as well as verbal instructions, which are reviewed frequently throughout the study. It is also important to factor into the design of the study the limitations of working with human volunteers. Studies that place unrealistic demands on subjects are likely to have compliance problems and a high attrition rate.

(10) *The experimental parumrters measured*—In addition to measuring changes in the mineral status (demineralization/remineralization), *in situ* model studies may include measuring fluoride uptake, plaque pH changes, plaque organic acid profiles, carbohydrate substrate

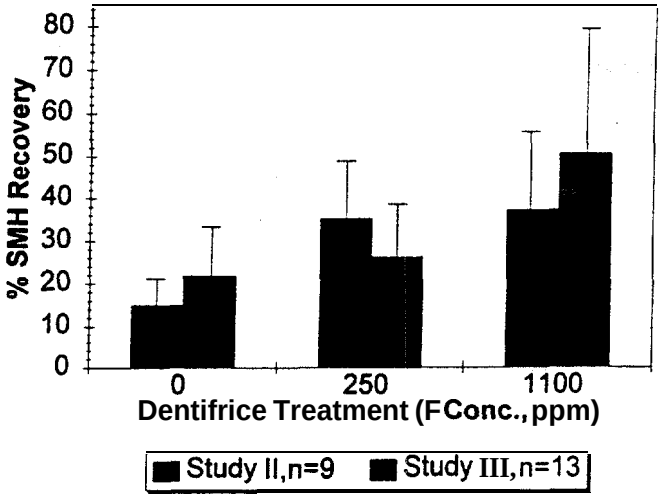

Fig. 9—Comparison of two different fluoride (F) dose-response studies involving the gauze-free remineralization model. The data are reported as mean % surface microhardness (SMH) recovery (remineralization). The bar represents the standard deviation.

clearance, salivary flow rate and composition, oral microbial counts, and substantivity of the test agent. The research objective(s) should determine which parameters are to be measured in an *in situ* study.

IN SITU MODEL VALIDATION

The major conclusion of the 1990 Consensus Conference on Intra-oral Models was the need for validation of *in situ* models for their potential as methods of evaluating the efficacy of fluoride dental products. While this present treatment of *in situ* models is intended to be much broader than the testing of fluoride agents, the fluoride dose-response data from clinical trials are the only solid link that we have to "natural" caries.

We have conducted several dose-response studies utilizing three different *in situ* models. Fig. 9 shows the results of two different studies using the gauze-free remineralization model. The only difference in the experimental design between the studies was that for Study II a bilateral design was used with enamel blocks held on the left and right sides of each subject's mandibular partial denture, with the results averaged. For Study III, enamel blocks were placed on only one side of each subject's mandibular partial denture. The 250-ppm-fluoride dentifrice treatment was significantly different from the 1100-ppm-fluoride treatment for Study III, but not for Study II. The comparison of these studies illustrates that the shape of the fluoride dose-response curve will be different when different study populations are used. This supports the need for internal validation of each *in situ* study.

Fig. 10 shows data from four different studies using the

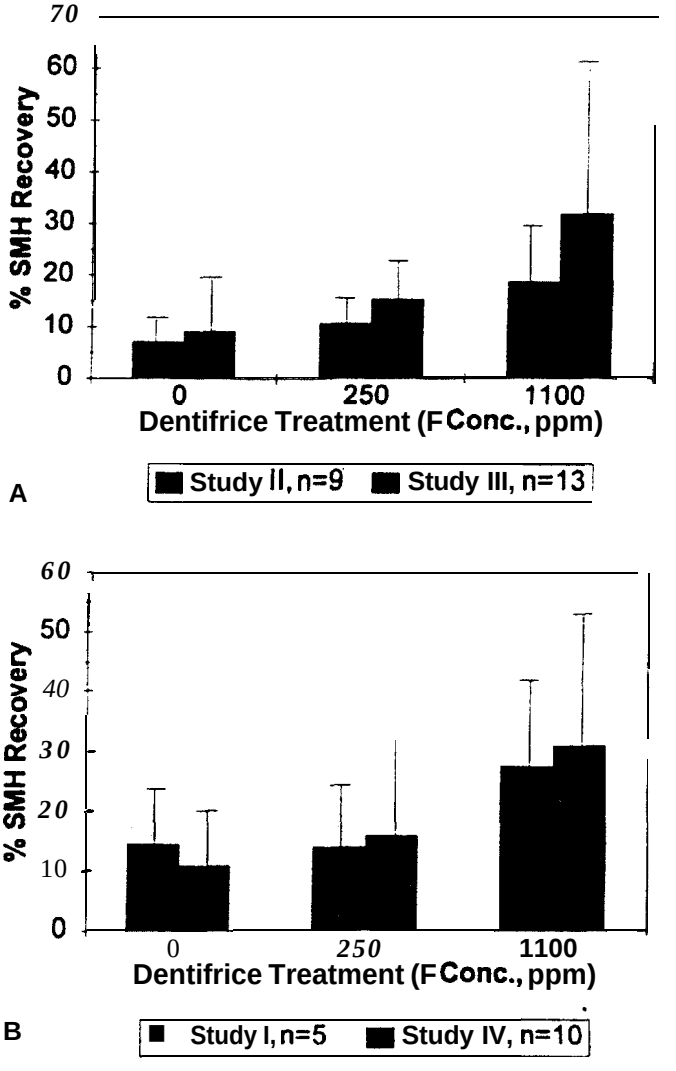

Fig. IO-Comparison of four different fluoride (F) dose-response studies involving the gauze-covered remineralization model. (a) Study II and III. (b) Study I and IV. The protocol was the same for all studies with the exception that for studies II and III a twice-daily extra-oral 10% sucrose challenge was added. The data are reported us mean % surface microhardness (SMH) recovery (remineralization). The bar represents the standard deviation.

gauze-covered remineralization model. Studies II and III involved the addition of a twice *per* day extra-oral 10% sucrose challenge, while Studies I and IV did not. In all other respects, the studies were the same. The 250-ppm-fluoride treatment was separated statistically from the 1100-ppm-fluoride treatment for each of the studies with the exception of Study I, due to the low number of subjects (n = 5). The dose-response curves for Studies I and IV are very similar; however, the dose responses for Studies II and III are different. The difference in the observed response in the latter

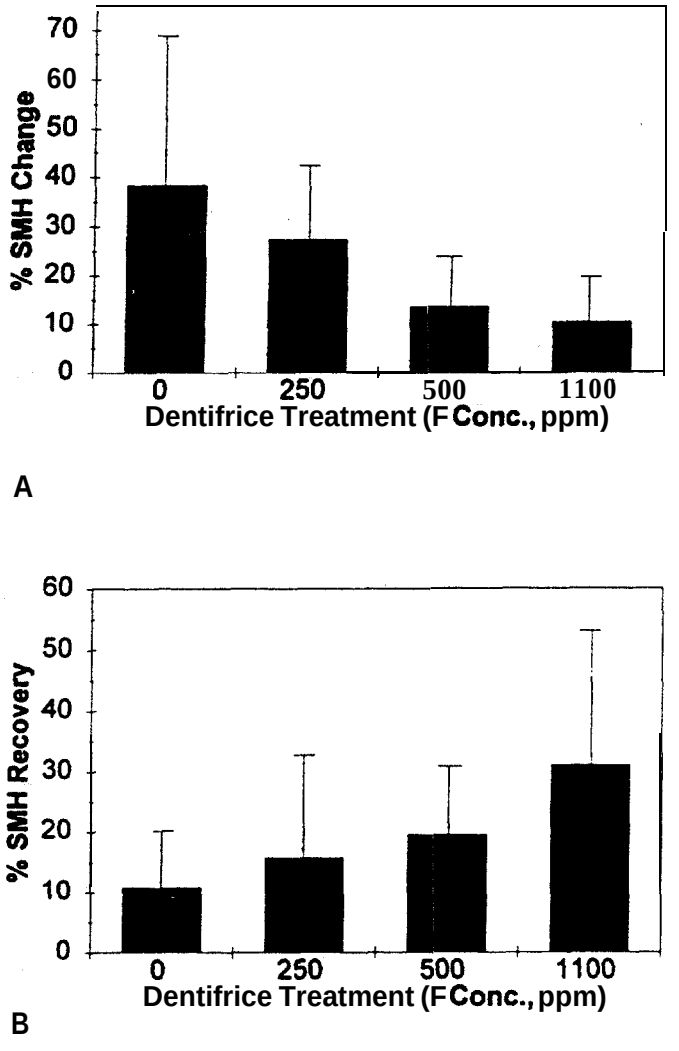

Fig. I l-Simultaneous comparison of the fluoride (F) dose responses of models with different hard tissue substrates (Study IV). (a) Gauze-covered demineralization model with sound bovine enamel. Data are reported as % surface microhardness (SMH) change (demineralization). (b) Gauze-covered remineralization model with surface-softened bovine enamel. Data are reported as % SMH recovery (remineralization).

two studies may be due to the addition of the sugar challenge to the model, which may accentuate differences in the response of subjects in the gauze-covered model.

Fig. 11 compares the fluoride dose responses of models with different hard tissue substrates. For the gauze-covered demineralization model, we used sound bovine enamel, and for the gauze-covered remineralization model, we used surface-softened bovine enamel. Both models demonstrated a fluoride dose response, with the demineralization model having an inverse relationship with fluoride concentration (Fig. lla), and the remineralization model having a direct relationship with fluoride concentration (Fig. 1 lb). It is interesting that enamel blocks placed in the mouth within millimeters of each other under identical experimental conditions can have radically different responses. This reaffirms the importance of the nature of the hard tissue substrate in the response of *in situ* models.

CONCLUSIONS

In situ caries models represent the most promising link that we have to "natural" caries, short of large-scale, well-conducted clinical trials. We must accept the reality that *in situ* models are in fact "models" that attempt to reproduce what occurs in clinical caries processes on a limited number of subjects. The success of an *in situ* model will be determined by its ability to maintain clinical relevance while controlling variation. The sources of experimental variation that are under the control of the investigator include: the physical design of the model, the hard tissue substrate and method of assessing mineral changes, and the clinical protocol.

The design and conduct of proper *in situ* model studies require a clear understanding of the caries process from both a mechanistic and a clinical standpoint, sound analytical support so that accurate and interpretable data can be obtained, and a knowledge of the limitations of working with human volunteers and how best to achieve subject compliance with research protocols. By taking this approach, we have been able to validate our *in situ* models based on fluoride dose response, as has been recommended by Proskin *et al.* (1992).

Based on this review of *in situ* caries models, I make the following recommendations:

(1) The design of *in situ* caries model systems must be carefully considered with regard to the objectives of the research, maximizing clinical relevance, minimizing variation, and maintaining high ethical standards.

(2) We must move toward standardization of the major experimental parameters: subject selection. physical design of models, hard tissue substrate and methods of assessment. and study design and clinical protocols.

(3) The major source of variation associated with *in situ* models should be of biological origin and not experimental origin. The main source of variation should come from differences in the biological responses of the subjects and not from variation arising from physical design of the model. the response of hard tissue substrates or the techniques of assessing mineral status, or uncontrolled delivery of test treatments.

(4) A combination of *in situ* caries model approaches may be necessary to achieve maximum predictive value for all possible clinical outcomes. It may be necessary to model caries with multiple hard tissue substrates, including sound enamel and surface-softened and subsurface lesions. There continues to be a need for comparative studies of the different *in situ* model systems.

(5) Internal fluoride dose-response controls should continue to be included in the design of *in situ* studies involving the evaluation of topical fluoride formulations.

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