December 14, 1999

Charles Ganley, M.D.
Director, Division of
Over-the-Counter Drug Products
Food and Drug Administration
9201 Corporate Boulevard, S205 (HFD-560)
Rockville, MD 20850

RE: Docket No 80N-0042 --Anticaries Drug Products
For Over-the-Counter Human Use

Dear Dr. Ganley,

This letter responds to FDA’s April 30, 1999 Feedback Letter in which the agency requests answers to questions as a part of an ongoing dialogue on biological testing methods in the Anticaries Final Monograph. Five copies of the submission are enclosed for the use of your staff. Two copies have been sent to the Dockets Management Branch.

**Action Requested:**

We request that the agency acknowledge in writing that the contemporary protocols, consistent with the Key Elements outlined in our feedback meeting background document submitted to the agency on March 12, 1999, are compliant procedures for final formulation testing of anticaries drug products. This matter is of major importance to members companies engaging in testing of fluoride-containing dentifrices. Should a working session help the agency come to a resolution in this matter, we would be happy to arrange such a session at the agency’s convenience.
Background:

The Joint Oral Care Task Group of the Consumer Healthcare Products Association (CHPA) and the Cosmetic, Toiletry, and Fragrance Association (CTFA) reviewed the protocols used to perform the required Anticaries Biological Testing of dentifrice drug products and submitted this review and the Key Elements of Anticaries Testing to the agency. A feedback meeting was held April 12, 1999 to discuss these Key Elements and during the meeting, questions were raised by the agency on the Task Group’s submission. These questions were communicated to the Task Group in writing on April 30, 1999.

Organization of Submission

The responses to these questions are provided here and are organized by biological test method. Each section lists FDA’s question cited in the April 30, 1999 Feedback Letter followed by the pertinent Key Element of Anticaries Testing described at the April 12, 1999 Feedback Meeting and the Task Group’s response to the question. These responses address only the concerns raised in FDA’s April 30, 1999 feedback letter and should be interpreted in conjunction with the full April 12, 1999 Feedback Meeting Background Document. The Task Group’s Background document is enclosed for easy reference when considering the responses to the questions. Copies of the study reports cited in this text are enclosed behind this letter as well as an alphabetical compilation of the references cited.

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1 As required by monograph Anticaries Drug Products for OTC Human Use and included in Docket No. 80N-0042.
1. Animal Caries Reduction Test

FDA's Question:
Individual comparative data assessing sodium fluoride, stannous fluoride and sodium monofluorophosphate reference standards compared to their negative controls in both 5-percent and 56-percent sucrose dietary studies should be provided.

Key Element of Animal Caries Testing Pertinent for Discussion:

Preconditioning of the animals must provide for 1) a cariogenic oral microflora, either naturally or through inoculation, 2) a cariogenic dietary challenge sufficient to promote caries, and 3) appropriate water source.

Task Group's Response:
Sodium fluoride (NaF)/silica, stannous fluoride (SnF₂)/silica, sodium monofluorophosphate (SMFP)/dical and sodium monofluorophosphate/silica have been compared to a fluoride-free negative control (Placebo) in both the 5-percent and 67-percent sucrose diets, as these levels represent the lowest and highest levels of sucrose currently included in the industry protocols.

The data are provided below:

<table>
<thead>
<tr>
<th>USP Reference Standard</th>
<th>% Reduction in Total Caries (Relative to Placebo)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5% Sucrose Diet*</td>
</tr>
<tr>
<td>NaF/silica</td>
<td>51</td>
</tr>
<tr>
<td>SnF₂/silica</td>
<td>44</td>
</tr>
<tr>
<td>SMFP/silica</td>
<td>30</td>
</tr>
<tr>
<td>SMFP/dical</td>
<td>25</td>
</tr>
</tbody>
</table>


Note: Data shared by CHPA/CTFA with FDA on 4/12/99 suggested comparisons between 5% and 56% sucrose diets had been made. The 56% data point presented was a typographical error. The level of sucrose in the studies presented at the 4/12/99 meeting were actually 67% sucrose. Therefore, the data that were presented represented the highest and lowest levels of sucrose currently in use in the industry protocols, 67% sucrose and 5% sucrose, respectively. The USP Reference Standard Dentifrices for the major fluoride salts included in the anticaries monograph were also tested in these two protocols.
Data in this table are derived from summary reports of each study. The table above presents Total Caries data collected from each study. Both the 5% and 67% sucrose diet models provide results that statistically separate all of the tested USP Reference Standards from Placebo. Identical batches of USP Reference Standards were included in each test.

These data demonstrate that whether a 5% or a 67% sucrose diet is used to meet the Key Element for Preconditioning, 1) the cariogenic challenge is sufficient to promote caries; and 2) the USP Reference Standards perform at a level that is statistically different from the placebo control. It is understood that each profile test must employ methods of statistical design and analysis, sufficient to assure that the experiment is valid (i.e., clinically proven USP Reference Standard is statistically significantly superior to a negative control) and that the test product is both significantly superior to the fluoride-free negative control and not significantly lower in performance than the USP Reference Standard. The models have been shown to be capable of detecting the anticaries benefits of clinically proven fluoride-containing dentifrices.

* * * * * *

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3 see Results, p. 3, Study # AC655A; and Table 1221A-6, p. 14, Study # 1221A of the enclosed study reports.
2. Enamel Solubility Reduction (ESR) Test

FDA's Question:

*The rationale for using a water or saliva diluent in the treatment regimen should be provided.*

Key Element ESR Testing Pertinent for Discussion:

*Treatment must provide a reproducible, controlled application of the dentifrice to the substrate. (Conditions controlled include dentifrice diluent/dilution and treatment time.)*

Task Group’s Response:

A diluent is needed to reduce the toothpaste to an applicable form. Both water and saliva dilute the paste to a similar consistency to that achieved in the mouth under brushing conditions. The agency has accepted the choice of either saliva or water as a diluent for enamel fluoride uptake (EFU) testing; FDA method #40 specified “the diluent can be either water or pooled saliva.” The choice of these diluents would bring ESR testing into alignment with the diluents specified for EFU testing. Use of a single diluent within a study and inclusion of an appropriate clinically proven USP reference standard will ensure that the key element of providing a controlled application of the dentifrice to the substrate is met.

Each profile test must employ methods of statistical design and analysis, sufficient to assure that the experiment is valid (i.e., clinically proven USP Reference Standard is statistically significantly superior to a negative control) and that the test product is both significantly superior to the fluoride-free negative control and not significantly lower in performance than the USP Reference Standard.

* * * * *
3. **Enamel Fluoride Uptake (EFU) Test**

**FDA's EFU Question A:**

*The benefits of allowing a range in lactic acid concentrations (0.025M – 0.1M) used for preconditioning should be described.*

**FDA's EFU Question C:**

*An explanation of Carbopol use should be provided.*

**Key Element of EFU Testing Pertinent for Discussion:**

*Preconditioning must minimize residual surface fluoride and prepare the substrate for fluoride incorporation.*

**Task Group’s Response:**

EFU Questions A and C are being answered together because the responses are interrelated. To mimic the *in vivo* cariogenic process in the mouth, artificially induced lesions are required to prepare the substrate for EFU testing. Artificially induced lesions have been successfully generated by a number of published techniques (Featherstone, 1981), all of which require a balance between the level of acid and the amount and type of surface protectant found in a solution. A surface protectant is needed in order to allow for the penetration of acids into the subsurface enamel, leaving the surface enamel structurally intact. This process is consistent with the natural process of demineralization, where the protein rich pellicle which is deposited onto tooth surfaces from salivary components provides a similar protective nature against potentially acidic environments.

Without such protection, acids generated by the breakdown of fermentable carbohydrates in the mouth would quickly erode and eventually destroy the teeth from the surface down into the subsurface regions. Artificial demineralization methods, such as Test Method 40, utilize MHDP (N-2-hydroxyethyl, methane hydroxy diphosphonate) as a surface protectant. The level of MHDP used is balanced with the level of acid to effect the development of artificial lesions over a relatively short period of time, leaving the tooth partially demineralized but structurally intact. MHDP is not a commercially available chemical. Other compounds have been
utilized to provide similar protection of the enamel surface during demineralization. One such compound that has been found to provide excellent protection of the enamel surface yet allow for the penetration of acids through the enamel surface is Carbopol, a synthetic, high molecular weight polymer (White, 1987) of cross-linked polyacrylic acid. Depending on the amount of Carbopol added to a solution, combined with the level of acid in the solution, the development of artificial lesions can be reproducibly controlled, providing a consistent substrate for testing.

Of primary importance in the development of artificial lesions is the balance between surface protectant (i.e. MHDP, carbopol, etc.) and acid, (i.e., lactic, acetic, etc.) along with the under saturation (i.e., calcium phosphate and pH) in the solution. Depending on the degree of under saturation, lesion development can be further tempered. In order to control the formation of artificial lesions with intact enamel surfaces, a balance of factors has been taken into account. The methods included in the CHPA/CTFA submission have been developed to provide reproducible, artificial lesions.

Reference for EFU Questions A & C


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FDA's EFU Question B:
An explanation of pH cycling and additional information regarding
the benefits of using this technique should be provided

Key Element of EFU Testing Pertinent for Discussion:
The treatment must provide reproducible conditions (diluent/dilution,
time, and frequency) of application of dentifrice to substrate.

Task Group's Response:
pH cycling models have been widely utilized by the dental scientific
community as well as the American Dental Association to test for enamel
fluoride uptake. The major difference between pH cycling and current FDA
method #40 is the enamel treatment procedure, with the cycling method using
multiple brief exposures to dentifrice, and Method 40 using a single 30
minute-long exposure. The pH cycling protocols used by different
investigators vary in detail, but all protocols contain the following 2 steps:

a. Enamel samples (sound or demineralized) are subjected to
multiple cycles of acid challenge, dentifrice treatment, and immersion
in remineralizing solutions. Alternating periods of acid challenge,
brief exposure to dentifrice slurries, and exposure to remineralizing
conditions are designed to simulate the exposure of natural teeth to
plaque acid, dentifrice, and salivary buffering and remineralization.

b. Enamel fluoride uptake as well as additional measurements
(e.g. mineral density, surface hardness) can be assessed.

As with the other current docketed EFU testing methods, pH cycling
permits assessment of fluoride uptake into enamel after exposure of
specimens to product treatment. In addition, the methods provide additional
information on other aspects of fluoride activity, (especially the ability of the
test materials to inhibit enamel demineralization and/or promote
remineralization) which can be useful during the dentifrice development process and thereby limiting the amount of enamel needed.

The pH cycling models have been shown to be capable of detecting the anticaries benefits of clinically proven fluoride-containing dentifrices. Use of the same treatment regimen within a study and inclusion of an appropriate clinically proven USP Reference Standard will ensure that the key element of providing reproducible conditions for all applications of dentifrice substrate is met. It is understood that each profile test must employ methods of statistical design and analysis, sufficient to assure that the experiment is valid (i.e., clinically proven USP Reference Standard is statistically significantly superior to a negative control) and that the test product is both significantly superior to the fluoride-free negative control and not significantly lower in performance than the USP Reference Standard.

References for EFU Question B


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FDA’s EFU Question D:

*The advantage of using the Microdrill for sampling should be explained.*

Key Element of EFU Testing Pertinent for Discussion:

*The evaluation of substrate must use quantitative, controlled sampling and valid chemical analyses to allow for comparison of treatments.*

Task Group’s Response:

The primary advantage of using the Microdrill for sampling is its nondestructive nature of sample collection. The microdrill technique abrades the tooth and collects the particles in the area being drilled. While enabling the analyst to take a controlled micro-sample for quantitative assessment of fluoride uptake into the specimen, the non-destructive nature of the technique allows for the remaining specimen to be available for other measurements, as desired, which may be used to provide additional scientific information regarding the nature and activity of anticaries agents.

In 1978, when the original protocols for assessing fluoride uptake into enamel were submitted, the principal method available for assessing enamel fluoride content was a method known as the “acid etch technique”. This method is described in the protocol of the original Docket Method #40. The acid etch method requires the serial removal of layers from each specimen included in the study. This results in the ultimate destruction of study samples. It was generally recognized by the research community that, along with assessing fluoride uptake into the teeth, the assessment of remineralization (or reversal) of early lesions was also of interest. Efforts were made to develop techniques that allowed for the non-destructive assessment of fluoride incorporation into enamel, which then permitted remineralization assessments to be made on the same specimen from which fluoride analyses had been made. Hence, the microdrill sampling technique was developed (Haberman, 1980 and Sakkab, 1984). Using the microdrill technique, the analyst is able to excise a controlled sample from each
 specimen included in a study. By measuring both the diameter and depth of the area sampled, then analyzing the fluoride content of the sample using an appropriately calibrated fluoride specific ion electrode, the analyst is able to calculate the amount of fluoride in the sample. Data are presented either as ppm F, μg of fluoride per square centimeter of surface sampled, or μg of fluoride per cubic centimeter of surface sampled. Protocols which utilize the microdrill technique commonly suggest sampling to a constant depth. As a constant term is used throughout each individual study, the reporting format of the data can vary. Converting between ppm fluoride and μg fluoride/cm of surface sampled is easily done using a proper mathematical conversion (Mobley, 1981).

While the microdrill technique was first discussed in the literature by Haberman et al. in 1980, a full description of the system was published by Sakkab et al. in 1984. Sakkab reported that the overall accuracy of both the microdrill technique and the acid etch technique are similar. The microdrill technique has since been used routinely in a number of University laboratories, including Oral Health Research Institute - Indiana University, Dows Institute - University of Iowa, and the Eastman Dental Center - University of Rochester in the United States as well as additional laboratories in Europe and Asia. Numerous publications (Stookey, 1985; Reintsens, 1985; White, 1986; White, 1987a; White, 1987b; Bowman, 1988a; Bowman, 1988b; White, 1991; Faller, 1995a; Faller, 1995b; Faller, 1997) in peer-reviewed journals have identified the microdrill technique as the method by which fluoride analyses were conducted. Each of these studies differentiate the levels of fluoride incorporated into the study specimens over the course of the study, demonstrating the ability of the method to accurately detect differences between clinically proven formulations (often a USP Reference Standard) and placebo. Thus, the literature supports the use of the microdrill technique.
Extensive use of the microdrill in the literature over the past two decades confirms its acceptance and use as a valid technique for meeting the Key Element for: "Evaluation of Substrate", as the technique has been shown to provide a "quantitative, controlled sampling...to allow for comparison of treatments”.

Models using the Microdrill technique have been shown to be capable of detecting the anticaries benefits of clinically proven fluoride-containing dentifrices. It is understood that each profile test must employ methods of statistical design and analysis, sufficient to assure that the experiment is valid (i.e., clinically proven USP Reference Standard is statistically significantly superior to a negative control) and that the test product is both significantly superior to the fluoride-free negative control and not significantly lower in performance than the USP Reference Standard.

**References for EFU Question D**


White, D.J. and Faller, R.V. Fluoride uptake from anticalculus dentifrices *in vitro*. *Caries Res.* 21 (1); 40-46, 1987a.


FDA’s EFU Question E:
An explanation of the reasons for substituting bovine enamel for human enamel should be provided. In addition, comparative fluoride uptake data from bovine and human enamel should be provided.

Key Element of EFU Testing Pertinent for Discussion:

The substrate must be a suitable source of dental enamel mineral.

Task Group’s Response:

Bovine enamel is chemically similar to human enamel, and it exhibits similar behavior in a variety of caries models (Mellberg, 1992). Bovine enamel is more easily obtained and handled than natural human teeth. In addition, bovine enamel does not present biohazard issues that are a concern when handling human tissues.

The data tabulated below were taken from two separate EFU studies run at Indiana University, one using human enamel, and one using bovine enamel. Both studies used identical protocols (method #40) with regard to enamel preparation, treatment, and evaluation. The results clearly show the comparability of the two substrates in the enamel fluoride uptake test

<table>
<thead>
<tr>
<th>(Mean ± SEM, N=12)</th>
<th>Δ Enamel Fluoride Content (post-treatment minus pre-treatment) [a larger number indicates greater enamel fluoride uptake]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative Control</td>
</tr>
<tr>
<td>Study #1 Human Enamel</td>
<td>28 ± 4 ppm</td>
</tr>
<tr>
<td>Study #2 Bovine Enamel</td>
<td>21 ± 4 ppm</td>
</tr>
</tbody>
</table>

The models have been shown to be capable of detecting the anticaries benefits of clinically proven fluoride-containing dentifrices. It is understood that each profile test must employ methods of statistical design and analysis, sufficient to assure that the experiment is valid (i.e., clinically proven USP Reference Standard is statistically significantly superior to a negative control) and that the test product is both significantly superior to the fluoride-free negative control and not significantly lower in performance than the USP Reference Standard.

Reference for EFU Question E

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Conclusions

These responses answer FDA’s questions raised at the April 12, 1999 Feedback Meeting. We hope that the agency will acknowledge in writing that the contemporary protocols, consistent with the Key Elements provided to the Agency in March 1999, are compliant procedures for final formulation testing of anticaries drug products. We also hope that we can continue the interactions to develop a simplified process for recognizing that requirements for biological testing can be met with future protocols consistent with the Key Elements.

Should you have any additional questions, please contact Patrice Wright at 202-429-3532 or Betsy Anderson at 202-331-1770.

Sincerely,

Patrice B. Wright, Ph.D.
Director, Pharmacology & Toxicology
Consumer Healthcare Products Association

Enclosures:

April 12, 1999 Feedback Meeting Background Document
Study Number AC655A: Rat Caries Evaluation of USP Standards using 5% Sucrose Model. Conducted by Procter & Gamble Company Health Care Research Center.
Study Number 1221A: Effect of Experimental Dentifrice on Caries Formation in the Rat. Conducted by Indiana University School of Dentistry Bioresearch Facility.
Study Number 121898: Comparison of the EFU performance using human and bovine enamel substrates. Conducted by Enamelon, Inc.
Alphabetical Compilation of Papers Cited in Text

cc: 2 copies to Dockets Management Branch
Consumer Healthcare Products Association
Cosmetic, Toiletry, and Fragrance Association
Joint Oral Care Task Group
Anticaries Biological Test Methods
April 12, 1999 Feedback Meeting Briefing Document

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Tab VI. Historical Background Information on Anticaries Biological Test Methods
Tab VII. Key Elements of Biological Testing of Fluoride Dentifrice Products
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   B. Key Elements for Contemporary Protocols
      1. Animal Caries Reduction Test Method
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      3. Enamel Fluoride Uptake Test Method
Tab VIII. Perspectives of the Experts in the Field of Anticaries Testing
Tab IX. Summary

Appendix A Contemporary Protocols for Animal Caries Reduction Testing
Appendix B Contemporary Protocols for Enamel Solubility Reduction Testing
Appendix C Contemporary Protocols for Enamel Fluoride Uptake Testing

1 The new name of the Nonprescription Drug Manufacturers Association pending official adoption by the membership on March 13, 1999.

March 12, 1999
Consumer Healthcare Products Association  
Cosmetic, Toiletry, and Fragrance Association  
Joint Oral Care Task Group  
Anticaries Biological Test Methods  
April 12, 1999 Feedback Meeting Briefing Document

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<th>Page #</th>
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<td>2</td>
<td>Overview of the Key Elements of Docket Protocols: Enamel Solubility Reduction Test Method</td>
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<td>3</td>
<td>Overview of the Key Elements of Docket Protocols: Enamel Fluoride Uptake Test Method</td>
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<td>Summary of Key Elements and Range of Protocol Parameters: Animal Caries Reduction Test Method</td>
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<td>Summary of Key Element Parameters of Contemporary Protocols for Animal Caries Reduction Test Method</td>
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<td>6</td>
<td>Summary of Key Elements and Range of Protocol Parameters: Enamel Solubility Reduction Test Method</td>
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<td>Summary of Key Elements Parameters of Contemporary Protocols for Enamel Solubility Reduction Test Method</td>
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<td>Summary of Key Elements and Range of Protocol Parameters: Enamel Fluoride Uptake Test Method</td>
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<td>Summary of Key Element Parameters of Contemporary Protocols for Enamel Fluoride Uptake Method</td>
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<tr>
<td>10</td>
<td>Dental Researchers Who Have Reviewed the Key Elements</td>
<td>58</td>
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Tab I

Meeting Agenda
Consumer Healthcare Products Association  
Cosmetic, Toiletry, and Fragrance Association  
Joint Oral Care Task Group  
Anticaries Testing Subgroup  

April 12, 1999 Feedback Meeting  

AGENDA  

I. Welcome and Introduction  
   A. Introductions by FDA and Industry  
   B. Purpose of the Meeting  
   C. Agreement on Agenda  

II. Rationale for the Requested Action  

III. Background on the Cariogenic Process  

IV. Mechanism of Action of Fluoride and Key Elements of Final Formulation Testing Methods  

V. Summary and Final Remarks  

VI. Discussion of Key Elements and Requested Action  

VII. Summary of Agreed Upon Action Items  

VIII. Conclusion  

IX. Adjournment

March 12, 1999
TAB II

Industry Representatives
Expected to Attend the April 12, 1999 Meeting

March 12, 1999
CHPA/CTFA Joint Oral Care Task Group
Anticaries Testing Subgroup

April 12, 1999 Feedback Meeting
Expected to Attend

Industry Representatives
Andrea (Andrea) Lewis Allan, Senior Marketing Attorney, Unilever United States, Inc.
Elizabeth (Betsy) H. Anderson, Esquire, Assistant General Counsel, The Cosmetic, Toiletry and Fragrance Association
Richard (Rich) K. Bourne, Ph.D., Vice President, Regulatory Affairs, Block Drug Company
R. Michael (Mike) Buch, Ph.D., Associate Director, Oral Health Care Liaison, SmithKline Beecham Consumer Healthcare
Lewis P. (Lew) Cancro, Industry Liaison Representative
Willie J. (Willie) Carter, Ph.D., Senior Scientist Research & Development, Block Drug Company
Greg (Greg) Collier, Ph.D., Section Head, The Procter & Gamble Company
Thomas J. (Tom) Donegan, Vice President-Legal and General Counsel, The Cosmetic, Toiletry and Fragrance Association
Robert (Bob) Faller, Senior Scientist, The Procter & Gamble Company
Geoffrey (Geoff) Forward, Ph.D., Oral Health Care R&D Category Head, SmithKline Beecham Consumer Healthcare
D. Scott (Scott) Harper, Ph.D., Section Director-Oral Care Technology Department, Warner-Lambert Company
Lori (Lori) Kumar, Ph.D., Director, Oral Care, Warner-Lambert Company
Gerald N. (Jerry) McEwen, Jr., Ph.D., J.D., Vice President-Science, The Cosmetic, Toiletry and Fragrance Association
Christine (Chris) Moorman, Regulatory Affairs Manager, The Procter & Gamble Company
Bruce (Bruce) Nelson, Senior Manager for Clinical Programs, Church & Dwight Co., Inc.
Barbara (Barbara) Popek, Assistant Director, Regulatory Affairs, SmithKline Beecham Consumer Healthcare
Peter (Peter) Ren, Ph.D., Senior Technical Associate, Colgate-Palmolive Company
R. William (Bill) Soller, Ph.D., Senior Vice President & Director of Science and Technology, Nonprescription Drug Manufacturers Association
Richard (Rich) Sullivan, Senior Technical Associate, Colgate-Palmolive Company

March 12, 1999
Lavada (Lavada) Watson, Manager, Regulatory Affairs, Colgate-Palmolive Company
Anthony E. (Tony) Winston, Vice President, Technology and Clinical Research, Enamelon, Inc.
Patrice B. (Patrice) Wright, Ph.D., Director, Pharmacology & Toxicology, Nonprescription Drug Manufacturers Association
Alex (Alex) Ziemkiewicz, Research Scientist, Unilever Home and Personal Care USA

Consultants
William H. (Bill) Bowen, Ph.D., Professor of Dentistry and Professor of Microbiology and Immunology, University of Rochester
George K. (George) Stookey, Ph.D., Director, Oral Health Research Institute, Indiana University School of Dentistry

March 12, 1999
Tab III

Definition of Terms Used in the Briefing Document
DEFINITIONS

The terminology differentiating protocols, test methods, and procedures is inconsistent in the Anticaries Monograph. For consistency, the terms used throughout this document are defined as:

**Test Method**
Any of the following biological test methods used to fulfill the requirements of the Anticaries Monograph: animal caries reduction, enamel solubility reduction, or enamel fluoride uptake.

**Key Elements**
The critical aspects of fluoride dentifrice biological testing which define the fundamental requirements of each test method. The **Key Elements** provide a methodological framework for the determination of animal caries reduction, enamel solubility reduction, and enamel fluoride uptake testing.

**Protocol**
A descriptive set of specific procedural steps to execute the test methods.

**Contemporary Protocols**
Protocols currently used to meet biological testing requirements of the Anticaries Monograph (including those not in the docket).
Tab IV

Discussion Topics and Requested Actions
Discussion Topics and Requested Actions

The CHPA/CTFA Joint Oral Care Task Task Group reviewed the protocols used to perform the required Anticaries Biological Testing of dentifrice drug products, contemporary protocols currently employed by manufacturers and research laboratories, and current state of the science to identify elements common among all protocols that are fundamental to each anticaries biological test method. These Key Elements, which are essential for properly designed protocols, are described in this briefing document under the headings: the principle of the test; the substrate or animal examined in the experiment; sample size and controls; preconditioning; treatment regimens; and evaluation of the substrate. At the feedback meeting, we plan to discuss the Key Elements of anticaries testing and contemporary Anticaries Biological Testing protocols.

Discussion Topics
We would like to reach agreement with the FDA on the following items:

A. The Key Elements describe the critical aspects of generally accepted fluoride dentifrice biological testing as found in the Anticaries Docket. (See Tab VII.A)

B. The contemporary protocols, not listed in the Docket, but utilized for anticaries testing are consistent with the Key Elements. (See Tab VII.B)

C. Contemporary protocols, consistent with the Key Elements, are compliant procedures for final formulation testing. (See Tab IX)

Requested Actions
1. We request that the agency acknowledge in writing that the contemporary protocols, consistent with the Key Elements, are compliant procedures for final formulation testing of anticaries drug products.

2. We request that the agency work with industry to develop a simplified process for recognizing that requirements for biological testing can be met with future protocols consistent with the Key Elements.

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2 As required by monograph Anticaries Drug Products for OTC Human Use and included in Docket No. 80N-0042.

March 12, 1999
Tab V

Summary Sheets of The Key Elements of the Anticaries Monograph Biological Test Methods
Summary Sheet of Key Elements of Biological Testing of Fluoride Dentifrice Products

Animal Caries Reduction

Principle and Purpose:
To compare the ability of a test dentifrice and a clinically proven USP Reference Standard to reduce dental carious lesions in animals undergoing cariogenic challenge. The pathogenesis of dental caries is essentially the same in all proposed animal models.

Animal (Substrate):
Animals are the substrate for this test. They must be caries susceptible, healthy, and easily treated. Rats with a natural or induced cariogenic oral microflora are the animals most widely utilized.

Sample Size and Controls:
The sample size must be adequate to meet the statistical requirements of the test.

Controls must include a clinically proven USP Reference Standard and an appropriate fluoride-free negative control.

Preconditioning:
Preconditioning of the animals must provide for 1) a cariogenic oral microflora either naturally or through inoculation, 2) a cariogenic dietary challenge sufficient to promote caries, and 3) an appropriate water source.

Treatment Regimen:
Test duration must be sufficient to produce adequate levels of caries. Dentifrice administration (frequency, applicator, and dilution) must be sufficient to demonstrate the effectiveness of a clinically proven USP Reference Standard vs. fluoride-free negative control.

Evaluation of Substrate:
The experiment must provide for accurate, reproducible visualization and enumeration of carious lesions.
Summary Sheet of Key Elements of Biological Testing of Fluoride Dentifrice Products

Enamel Solubility Reduction

Principle and Purpose:
To compare the ability of a test dentifrice and a clinically proven USP Reference Standard to reduce the solubility of enamel during acid challenge.

Substrate:
The substrate must be a suitable source of dental enamel mineral.

Sample size and controls:
The sample size must be adequate to meet the statistical requirements of the test.

Controls must include a clinically proven USP Reference Standard and an appropriate fluoride-free negative control.

Preconditioning:
Preconditioning must minimize residual fluoride content and prepare the enamel mineral for the fluoride incorporation, which is required to reduce enamel solubility during acid challenge.

Treatment Regimen:
Treatment must provide a reproducible, controlled application of the dentifrice to the substrate. (Conditions controlled include diluent, dilution and treatment time).

Evaluation of Substrate:
The evaluation must provide a reproducible, controlled acid challenge to the substrate. Etch solutions must be quantitated for analytes indicative of enamel dissolution by valid analytical methods.

March 12, 1999
Summary Sheet of Key Elements of Biological Testing of Fluoride Dentifrice Products

Enamel Fluoride Uptake

Principle and Purpose:
To compare the ability of a test dentifrice and a clinically proven USP Reference Standard to effect the fluoride incorporation into demineralized enamel.

Substrate:
The substrate must be a suitable source of dental enamel mineral.

Sample Size and Controls:
The sample size must be adequate to meet the statistical requirements of the test.

Controls must include a clinically proven USP Reference Standard and an appropriate fluoride-free negative control.

Preconditioning:
Preconditioning must minimize residual surface fluoride and prepare the substrate for fluoride incorporation.

Treatment Regimen:
The treatment must provide reproducible conditions (diluent/dilution, time, and frequency) for application of dentifrice to substrate.

Evaluation of Substrate:
The evaluation of substrate must use quantitative, controlled sampling and valid chemical analyses to allow for comparison of treatments.

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Tab VI

Historical Background Information on Anticaries
Biological Test Methods

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Historical Background Information

The review of ingredients for inclusion in the monograph for anticaries drug products for over-the-counter use led to the general recognition of the safety and effectiveness of three fluoride compounds: sodium fluoride, stannous fluoride, and sodium monofluorophosphate. During the course of the review, industry identified the potential of these agents to interact with dentifrice components (predominantly the abrasive system of the dentifrice) and provided eight compatible fluoride abrasive combinations that had been established as effective in clinical trials. The FDA published the combinations in the Final Monograph as USP Reference Standards against which fluoride dentifrices would be tested to assure their effectiveness. To ensure this process, manufacturers agreed to provide the FDA and the USP with clinically established anticaries reference standards, the biological test methods, and protocols to be employed as predictors of clinical effectiveness in the Anticaries Final Monograph.

In providing these predictors, industry assured that the performance of new fluoride dentifrices could be compared to clinically tested USP Reference Standards. Specifications for the physical and chemical properties of the combinations and their stability profiles were also provided by dentifrice manufacturers for inclusion in the Final Monograph to assure that fully potent products could be manufactured by following the provided formulary information of clinically effective fluoride dentifrices.

Thus, to assure that the effectiveness of OTC fluoride dentifrices is not compromised, three principles exist to make the Anticaries Monograph a practical working document:

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1. The physical and chemical properties of the dentifrice must initially match established specifications.
2. The available fluoride of the product must match a given profile over the shelf-life of the product.
3. The fluoride dentifrice must meet the biological testing requirements of the monograph using clinically proven USP Reference Standards as the internal control.

Today, some 20 years after this program was initiated, the members of the CHPA/CTFA Joint Oral Care Task Group can report that fluoride dentifrices manufactured by these principles are in compliance with the Final Monograph. Currently marketed products meet all of the safety and efficacy requirements. While the test methods have not changed, an evolution of the protocols has occurred. The use of alternate buffering systems, sampling techniques, analyte resolution, etc., have, in many cases, resulted in enhancements of the contemporary protocols.

We have reviewed the contemporary protocols used to conduct these tests and determined that these tests confirm the effectiveness of fluoride dentifrice products. All contemporary protocols encompass the essential Key Elements that define the test method and are able to demonstrate the effectiveness of a clinically proven USP Reference Standard vs. a negative control.

The Key Elements are the critical aspects of fluoride dentifrice biological testing which define the fundamental requirements of each test method. The Key Elements provide a methodological framework for the determination of animal caries reduction, enamel solubility reduction, and enamel fluoride uptake.

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Tab VII

Key Elements of Biological Testing of Fluoride Dentifrice Products
A. Key Elements of FDA Biological Test Methods

Discussion Topic A:
The Key Elements describe the critical aspects of generally accepted fluoride dentifrice biological testing as found in the Anticaries Docket.

During the anticaries rulemaking process, biological test methods were provided by the industry and subsequently accepted by the OTC Advisory Panel and the Agency. The protocols outlined procedures for test methods developed by dental researchers and manufacturers that were historically used in dentifrice development programs to assure fluoride availability prior to clinical evaluation. The test methods constitute methods that have a history of association and correlation with anticaries clinical trials and were accepted by the Food and Drug Administration into the monograph.

Any scientific test method, by its nature, has a set of essential elements that make it a valid test. Generally, these are the principle and purpose of the test, the controls used, the substrate on which the test is performed, the sample size, the pretreatments used, the treatment regimen employed, and the unbiased evaluation of the substrate. The CHPA/CTFA Joint Oral Care Task Group reviewed the diverse protocols in the Anticaries docket and confirmed that the protocols all share parameters defined by these elements. These are the Key Elements. For each anticaries test method, there are protocols in the docket that demonstrate a product's performance is at least as good as a clinically effective product (i.e., USP Reference Standard) and significantly more effective than a fluoride-free negative control.

The following tables, one for each test method (Animal Caries Reduction—Table 1, Enamel Solubility Reduction – Table 2, and Enamel Fluoride Uptake...
- Table 3, provide a summary of the protocols currently in the docket. The parameters of each protocol are organized under the Key Element headings: principle and purpose; substrate; sample size and controls; preconditioning; treatment regimen; and evaluation of substrate.

For each Key Element parameter, the agency accepted a wide latitude in the protocols. However, for the three test methods, each protocol is consistent with the Key Elements.
Table 1: Overview of the Key Elements of Docket Protocols: Animal Caries Reduction Test Method

**PRINCIPLE AND PURPOSE OF TEST METHOD:** To compare the ability of a test dentifrice and a clinically proven USP Reference Standard to reduce dental carious lesions in animals undergoing cariogenic challenge. The pathogenesis of dental caries is essentially the same in all animal models.

<table>
<thead>
<tr>
<th>Animal (Substrate)</th>
<th>Sample Size and Controls</th>
<th>Preconditioning</th>
<th>Treatment Regimen</th>
<th>Evaluation of Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FDA #37 Protocol #1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wistar Rat</td>
<td>22-23 days</td>
<td>20 per group</td>
<td>DeH₂O/ Diet #469 with 63% sucrose</td>
<td>Resident Microflora</td>
</tr>
<tr>
<td>Osborne/ Mendel Rat</td>
<td>22-24 days</td>
<td>16-20 / group</td>
<td>Tap H₂O/ Diet MIT 9100 with 56% sucrose</td>
<td>Resident Microflora</td>
</tr>
<tr>
<td><strong>FDA #38 Protocol #4</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osborne/ Mendel or Cara Rat</td>
<td>24 days</td>
<td>Not stated</td>
<td>Tap H₂O/ Diet #2000a with 56% sucrose</td>
<td>Inoculated G. mutans OMZ176 A. viscosus OM210G</td>
</tr>
<tr>
<td><strong>FDA #39 Protocol #13</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiple strains</td>
<td>22-24 days old</td>
<td>16-20 or not stated</td>
<td>Various water sources and diets (with 56-63% sucrose)</td>
<td>Resident or inoculated cariogenic microflora</td>
</tr>
</tbody>
</table>

**Key Element Description**

- **Animals must be caries susceptible, healthy, and easily treated. Rats with a natural or induced cariogenic oral microflora are the animal most widely utilized.**
- **The sample size must be adequate to meet the statistical requirements of the test.**
- **Controls are the same for each experiment and must include a clinically proven USP Reference Standard and an appropriate fluoride free negative control.**
- **Preconditioning of the animals must provide for 1) a cariogenic oral microflora, either naturally or through inoculation, 2) a cariogenic dietary challenge sufficient to promote caries, and 3) an appropriate water source.**
- **Test duration must be sufficient to produce adequate levels of caries. Dentifrice administration (frequency, applicator, and dilution) must be sufficient to demonstrate clinically proven USP Reference Standard effectiveness vs. negative control.**
- **The evaluation of the substrate must provide for accurate, reproducible visualization and enumeration of carious lesions.**

---

For each Key Element parameter, the agency accepted a wide latitude in the protocols. However, for the three test methods, each protocol is consistent with the Key Elements.

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Table 2: Overview of the Key Elements of Docket Protocols: Enamel Solubility Reduction Test Method

**PRINCIPLE AND PURPOSE OF THE TEST METHOD:** To compare the ability of a test dentifrice and a clinically proven USP Reference Standard to reduce the solubility of enamel during acid challenge.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sample Size and Controls</th>
<th>Preconditioning</th>
<th>Treatment Regimen</th>
<th>Evaluation of Substrate</th>
<th>Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDA # 33 Protocol 14</td>
<td>Human molar crowns (6/set)</td>
<td>4</td>
<td>etch in lactate buffer</td>
<td>Water 1:3 supernatant</td>
<td>Pre- and Post Treatment etches for 15 minutes at 37°C in lactate buffer</td>
</tr>
<tr>
<td>FDA # 34 Protocol 20</td>
<td>Human molar crowns (4/set)</td>
<td>3</td>
<td>etch in lactate buffer</td>
<td>Water 1:3 slurry</td>
<td>Pre- and post-treatment etches for 15 minutes at 37°C in lactate buffer</td>
</tr>
<tr>
<td>FDA # 35 Protocol 21</td>
<td>Hydroxyapatite disc or enamel chips</td>
<td>Not Stated</td>
<td>Soak in water (Hydroxyapatite discs) or Etch in acetate buffer (enamel chips)</td>
<td>Water 1:3 slurry</td>
<td>Post-treatment etch in acetate buffer for 8 minutes (chips) or 1 hour (discs) at 37°C</td>
</tr>
<tr>
<td>FDA # 36 Protocol 22</td>
<td>Powdered human enamel</td>
<td>1</td>
<td>Powdering</td>
<td>Water 1:3 supernatant</td>
<td>Post treatment etch in acetate buffer for 45 minutes at 37°C</td>
</tr>
<tr>
<td>Range of Key Element Parameters within Docket Protocols</td>
<td>Human enamel crowns, hydroxyapatite discs, Enamel chips or powdered enamel</td>
<td>Not Stated, 1 to 4</td>
<td>Lactic acid etch, water soak, acetic acid etch or powdering</td>
<td>Water 1:3 supernatant or slurry</td>
<td>Pre- and post-treatment or just post-treatment alone etches in lactate or acetate buffer for 8 to 60 minutes.</td>
</tr>
</tbody>
</table>

**Key Element Description**

- **Substrate must be a suitable source of dental enamel mineral.**
- **Sample size must be adequate to meet the statistical requirements of the test.**
- **Controls must be the clinically proven USP Reference Standard and an appropriate negative control.**
- **Preconditioning minimizes residual fluoride content and prepares enamel for fluoride incorporation that is required to reduce enamel solubility during acid challenge.**
- **Treatment must provide a reproducible, controlled application of the dentifrice to the substrate. Conditions controlled include dentifrice diluent/dilution and treatment time.**
- **The evaluation of the substrate must provide a reproducible, controlled acid challenge to the substrate. Etch solutions must be quantitated for analytes indicative of enamel dissolution by valid analytical methods.**

---

4 For each Key Element parameter, the agency accepted a wide latitude in the protocols. However, for the three test methods, each protocol is consistent with the Key Elements.

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Table 3: Overview of the Key Elements of Docket Protocols: Enamel Fluoride Uptake Test Method

**PRINCIPLE AND PURPOSE OF THE TEST METHOD:** To compare the ability of a test dentifrice and a clinically proven USP Reference Standard to affect the fluoride incorporation into demineralized enamel.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sample Size and Controls</th>
<th>Preconditioning of Samples</th>
<th>Treatment Regimen</th>
<th>Evaluation of Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDA #40 Protocol 25</td>
<td>Human Enamel (intact)</td>
<td>#/group not stated</td>
<td>Etch, 2M HClO₄</td>
<td>Pooled human saliva or water supernatant</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.025M lactic + 0.0002M MHDP</td>
<td>1:3</td>
</tr>
<tr>
<td>FDA #41 Protocol 34</td>
<td>Human Enamel (intact)</td>
<td>20/group</td>
<td>Grind, polish, etch</td>
<td>Deionized water slurry</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HClO₄ etch</td>
<td>1:3</td>
</tr>
<tr>
<td>FDA #42 Protocol 37</td>
<td>Human Enamel (powdered)</td>
<td>#/group not stated</td>
<td>Powdering</td>
<td>Synthetic saliva supernatant</td>
</tr>
<tr>
<td>FDA #43 Protocol 38</td>
<td>Human Enamel (powdered)</td>
<td>#/group not stated</td>
<td>Powdering</td>
<td>Distilled water supernatant</td>
</tr>
<tr>
<td>Range of Key Element Parameters within Docket Protocols</td>
<td>Intact or powdered human enamel</td>
<td>Not stated - 20/group</td>
<td>Etch, grind, polish, powder</td>
<td>Human or synthetic saliva, water, slurry or supernatant</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lactic acid, MHDP, perchloric or NA</td>
<td>not stated - 1:3</td>
</tr>
</tbody>
</table>

**Key Element Description**

- **The substrate must be a suitable source of dental enamel mineral**
- **The sample size must be adequate size to meet the statistical requirements of the test**
- **The controls are the same for each experiment and must include a clinically proven USP Reference Standard and an appropriate fluoride free negative control.**
- **Preconditioning must minimize residual surface fluoride and prepare the enamel for fluoride incorporation.**
- **Treatment must provide reproducible conditions (diluent/dilution, time, and frequency) for application of dentifrice to substrate.**
- **The evaluation of the substrate must use quantitative, controlled sampling and valid chemical analyses to allow for comparisons of treatments.**

For each Key Element parameter, the agency accepted a wide latitude in the protocols. However, for the three test methods, each protocol is consistent with the Key Elements.

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B. Discussion of the Key Elements for Contemporary Protocols

Discussion Topic B:
The protocols currently utilized for anticaries testing, not listed in the Docket, are consistent with the Key Elements.

Since 1978, there has been an evolution of the anticaries biological test protocols as a result of the efforts of a number of dental researchers who have made contributions to help better understand the caries process. While the Key Elements of anticaries testing have not changed, contemporary protocols reflect changing circumstances beyond the control of investigators and procedures that decrease variability or enhance sensitivity and reliability of the protocol.

The CHPA/CTFA Joint Oral Care Task Group believes that it is prudent to expand the boundaries of existing protocols in the docket to include protocol enhancements. These contemporary protocols reflect current scientific practices, are consistent with the Key Elements, and are not fundamentally different from protocols submitted during the rulemaking process. The Key Elements of each test method provide a framework for evaluating protocols to ensure that enhancements do not change the fundamental principles of anticaries testing and that a new product performs at least as good as a clinically proven product (i.e., USP Reference Standard). In the following pages, the Key Elements for each test method are described in detail. The general description of the Key Elements (as listed under Tab V) is

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6 For example, discontinued availability of reagents, instruments or rat strains
7 For example, better sampling techniques and analytical methods that provide better resolution of the analytes.

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highlighted in a box before the detailed discussion of the Key Element.

Additionally, it is understood that each profile test must employ methods of statistical design and analysis, sufficient to assure that the experiment is valid (i.e., clinically proven USP Reference Standard is statistically significantly superior to a negative control) and that the test product is both significantly superior to the fluoride-free negative control and not significantly lower in performance than the USP Reference Standard.
1. Key Elements of the Animal Caries Reduction Test Method

Animal caries testing of dentifrice products is part of the required testing mandated in the OTC Anticaries Drug Products Final Monograph, [21 CFR §355.70]. Animal caries models to evaluate the cariostatic potential of fluoride-containing dentifrices have been well established. The models have been shown to be capable of detecting the anticaries benefits of clinically proven fluoride-containing dentifrices.

Principle and purpose:

To compare the ability of a test dentifrice and a clinically proven USP Reference Standard to reduce dental carious lesions in animals undergoing cariogenic challenge. The pathogenesis of dental caries is essentially the same in all proposed animal models.

The basic principle of the animal caries reduction test is to measure the caries-inhibiting properties of fluoride-containing dentifrices in vivo. The remaining Key Elements set forth below provide a methodological framework for the determination of animal caries reduction that incorporates best current practices and an inherent ability to maximize the use of existing animal caries methods.

Substrate

Animals are the substrate for this test. They must be caries susceptible, healthy, and easily treated. Rats with a natural or induced cariogenic oral microflora are the animals most widely utilized.
While the caries process has also been studied in primates and hamsters, the rat has been the model of choice for decades. The rat model represents a true caries disease process due to the (1) vulnerability of the tooth to caries attack after eruption, (2) the use of a cariogenic diet, and (3) the presence of an established cariogenic microflora in the rats’ oral cavity.

The predominant rat strain reported for the animal caries model is the Sprague-Dawley. This strain has been shown to be hardy, caries susceptible, tolerant to treatment and inoculation of cariogenic bacterial strains, easy to handle, and widely available commercially. Wistar, Osborne-Mendel, and other strains have also been successfully used.

The age of the rats at study start is timed to follow shortly after eruption of the first molars, as this has an effect on establishing a cariogenic oral microflora and upon the susceptibility of the rats’ teeth to caries. Study start ages ranging from 20-24 days have been found to result in adequate levels of caries.

Animals should be treated in accordance with current standards of animal husbandry as specified in current United States Department of Agriculture (USDA) guidelines and American Association of Laboratory Animal Science (AALAS) requirements.
Sample size and controls:

The sample size must be adequate to meet the statistical requirements of the test.

Controls must include a clinically proven USP Reference Standard and an appropriate fluoride-free negative control.

The number of animals in each treatment group is determined by the individual model and must be adequate to meet the statistical requirements of the test. The number of animals used in these studies to discriminate between a clinically proven USP Reference Standard and a fluoride-free negative control will vary depending on the level and uniformity of the caries rate among the animals. Balancing of litters across treatment groups is generally practiced to reduce variability.

Controls used in each study must be a clinically proven USP Reference Standard (the positive control) and a fluoride-free negative control. The positive control must contain the same anticaries active as the test product.

Preconditioning:

The test animals must have a cariogenic oral microflora capable of causing dental caries in the presence of a cariogenic...
(sucrose-containing) diet. Some investigators maintain rat colonies in which the animals are known to harbor indigenous populations of cariogenic microorganisms. Other investigators superinfect the young rats with a known cariogenic strains to ensure that the test animals have a cariogenic oral microflora adequate to produce a disease state. The predominant bacterial species used to inoculate the animals are *Streptococcus sobrinus*, *Streptococcus mutans*, and *Actinomyces viscosus*. Inoculating the mouth of the rodent with an active culture on repeated days prior to the treatment phase of the test is usually successful. The presence of viable cariogenic bacteria in the mouth of the test animals should be confirmed during the course of the study.

The cariogenic diet must support the health of the animals and provide a sufficiently rigorous cariogenic challenge in the form of a fermentable carbohydrate. Sucrose is the cariogenic sugar of choice. Cariogenic diets used in the rat model have varied in their sucrose content, and sucrose has also been added to the drinking water. The state of the science suggests that variations in diet composition are not of material consequence as long as the diet fulfills its primary purpose of promoting caries.
Treatment Regimen:

The duration of the study should be adequate to ensure sufficient caries levels to provide statistical differentiation among treatment groups. Using current technology, successful models have been established wherein the duration of treatment varied from 3 to 6 weeks.

The rats can be treated with undiluted dentifrice or dentifrice slurry. Dentifrice slurries are generally diluted in the range of 50% (w/w) or less. The application is made with a suitable applicator such as a cotton swab. Treatment times and frequencies in the test procedure should assure adequate ability of the model to detect a significant difference between the clinically proven USP Reference Standard and the fluoride-free negative control.

Evaluation of Substrate:

The test procedures in the docket use caries visualization techniques ranging from scoring unstained teeth to using stains that enhance caries visualization. Lesion visualization

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procedures that use a variety of staining procedures (e.g. silver nitrate, Schiff's reagent, murexide, or merbromine), UV light, or a magnifying lens to enhance the ability of the scorer to visualize carious lesions are appropriate.

The scoring systems used in rat caries models enumerate coronal caries levels. The Keyes index as well as the Larson-modified Keyes index, the Francis and Konig scoring methods have been used to characterize the incidence and severity of rat caries. The total caries score should be the primary efficacy variable.

All scoring must be conducted on a blind basis. Treatment groups should be coded. However, assessment of examiner variability is optional. The mandatory information to be obtained includes caries incidence and caries severity; optional information includes: body weight, mortality, and examiner reliability.

**SUMMARY OF THE KEY ELEMENTS OF ANIMAL CARIES REDUCTION**

Contemporary protocols for animal caries testing can be found in Appendix A (numbered 1-13). Each contemporary protocol meets the requirements of the Key Elements. These protocols have been successfully used for the evaluation of marketed product.

Table 4 compares the Key Elements range of parameters within the docket protocols (alone) and the contemporary protocols. Generally, the range of parameters within each Key Element is similar in the

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contemporary protocols and the docket protocols. While the Key Elements of anticaries testing have not changed, contemporary protocols reflect changing circumstances beyond the control of investigators\textsuperscript{8} and procedures that decrease variability or enhance sensitivity and reliability of the protocol\textsuperscript{9}.

Table 5 provides a detailed comparison of the range of parameters within the Key Elements for all of the contemporary protocols used. Following the table is a comment on experimental parameters of contemporary protocols that are outside the range of the docket protocols. All protocols encompass the Key Elements, and the enhancements do not bias the outcome of the test. In addition, each profile test must employ methods of statistical design and analysis, sufficient to assure that the experiment is valid (i.e., clinically proven USP Reference Standard is statistically significantly superior) and that the test product is both significantly superior to the fluoride-free negative control and not significantly lower in performance than the USP Reference Standard.

\textsuperscript{8} For example, discontinued availability of reagents, instruments or rat strains
\textsuperscript{9} For example, better sampling techniques and analytical methods that provide better resolution of the analytes.

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Table 4: Summary of Key Elements and Range of Protocol Parameters: Animal Caries Reduction Test Method

**PRINCIPLE AND PURPOSE OF TEST METHOD:** To compare the ability of a clinically proven USP Reference Standard with a test dentifrice to prevent development of dental carious lesions in animals. The pathogenesis of dental caries is essentially the same in all proposed animal models.

<table>
<thead>
<tr>
<th>Key Elements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal (Substrate)</td>
</tr>
<tr>
<td>Sample Size and Controls</td>
</tr>
<tr>
<td>Preconditioning</td>
</tr>
<tr>
<td>Treatment Regimen</td>
</tr>
<tr>
<td>Evaluation of Substrate</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Key Element Description</th>
<th>Animal Strain: Wistar, Osborne/Mendel, Cara rats or Sprague-Dawley rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at Study Initiation:</td>
<td>22-24 days.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Key Element Description</th>
<th>Group sizes range from 16-20 or is not stated</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Key Element Description</th>
<th>Water Source: DI H2O, 5% - 10% Sucrose H2O, or Tap H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cariogenic Diet:</td>
<td>Diets include sucrose levels from 56-63%</td>
</tr>
<tr>
<td>Cariogenic microflora:</td>
<td>superinfection with S. mutans, A. viscosus or resident pathogens.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Key Element Description</th>
<th>Water Source: DI H2O, 5% - 10% Sucrose H2O, or Tap H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cariogenic Diet:</td>
<td>Diets include sucrose levels from 56-63%</td>
</tr>
<tr>
<td>Cariogenic microflora:</td>
<td>superinfection with S. sobrinus/mutans or resident pathogen.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Key Element Description</th>
<th>Water Source: DI H2O, 5% - 10% Sucrose H2O, or Tap H2O</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
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<tr>
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<tr>
<td>Cariogenic microflora:</td>
<td>superinfection with S. sobrinus/mutans or resident pathogen.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Key Element Description</th>
<th>Water Source: DI H2O, 5% - 10% Sucrose H2O, or Tap H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cariogenic Diet:</td>
<td>Diets include sucrose levels from 56-63%</td>
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<tr>
<td>Cariogenic microflora:</td>
<td>superinfection with S. sobrinus/mutans or resident pathogen.</td>
</tr>
</tbody>
</table>

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<thead>
<tr>
<th>Key Element Description</th>
<th>Water Source: DI H2O, 5% - 10% Sucrose H2O, or Tap H2O</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cariogenic Diet:</td>
<td>Diets include sucrose levels from 56-63%</td>
</tr>
<tr>
<td>Cariogenic microflora:</td>
<td>superinfection with S. sobrinus/mutans or resident pathogen.</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Key Element Description</th>
<th>Water Source: DI H2O, 5% - 10% Sucrose H2O, or Tap H2O</th>
</tr>
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<tbody>
<tr>
<td>Cariogenic Diet:</td>
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</tr>
<tr>
<td>Cariogenic microflora:</td>
<td>superinfection with S. sobrinus/mutans or resident pathogen.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Key Element Description</th>
<th>Staining/Sectioning: Substrate is unstained or stained with silver nitrate or Schiff's reagent sufficient to permit adequate visualization of incipient or gross lesions. Samples are hemi-sectioned, longitudinally cut, or multiply sectioned.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scoring System/Endpoints:</td>
<td>Briner/Francis, Keyes, Shrestha/Keyes, Keyes/Larson and Konig systems measuring incipient, or gross lesions or both.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Key Element Description</th>
<th>Staining/Sectioning: Substrate is unstained or stained with silver nitrate, murexide, and/or merbromine, sufficient to permit adequate visualization of incipient or gross lesions. Samples are hemi-sectioned, longitudinally cut, or multiply sectioned.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scoring System/Endpoints:</td>
<td>Briner/Francis, Keyes, Shrestha/Keyes, Keyes/Larson and Konig systems measuring incipient, or gross lesions or both.</td>
</tr>
<tr>
<td>Protocol Number</td>
<td>Animal (Substrate)</td>
</tr>
<tr>
<td>-----------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>1 FDA #37</td>
<td>Wistar rat</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
</tr>
<tr>
<td>4</td>
<td>Sprague Dawley rat</td>
</tr>
<tr>
<td>5</td>
<td>&quot;</td>
</tr>
<tr>
<td>6</td>
<td>&quot;</td>
</tr>
<tr>
<td>7</td>
<td>&quot;</td>
</tr>
<tr>
<td>8</td>
<td>&quot;</td>
</tr>
<tr>
<td>9</td>
<td>&quot;</td>
</tr>
<tr>
<td>10</td>
<td>&quot;</td>
</tr>
<tr>
<td>11 FDA #38</td>
<td>Osborne/ Mendel rat</td>
</tr>
<tr>
<td>12</td>
<td>Osborne/ Mendel (Resident Colony)</td>
</tr>
<tr>
<td>13</td>
<td>Osborne/ Mendel Or Cara</td>
</tr>
</tbody>
</table>
### Key for Notes in Table 5

<table>
<thead>
<tr>
<th>Key</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Each Protocol meets the requirements of the Key Elements as described in Table 4. These protocols have been successfully used for evaluation of at least one marketed product.</td>
</tr>
<tr>
<td>b</td>
<td>Controls are the same for each experiment and must include a clinically proven USP Reference Standard and an appropriate fluoride free negative control.</td>
</tr>
<tr>
<td>c</td>
<td>Twice daily, 5 days/week, for 3 weeks</td>
</tr>
<tr>
<td>d</td>
<td>This diet has been shown to provide an adequate cariogenic challenge.</td>
</tr>
<tr>
<td>e</td>
<td>Sprague Dawley strain is thought to be derived from the Wistar strain.</td>
</tr>
<tr>
<td>f</td>
<td>Using younger rats increases the consistency of establishing a cariogenic microflora, especially when infecting with exogenous cariogenic bacteria.</td>
</tr>
<tr>
<td>g</td>
<td>Increased group size (compared to the original docket protocol) raises statistical power and thereby the probability that the experiment will meet its statistical requirements.</td>
</tr>
<tr>
<td>h</td>
<td><em>S. Sobrinus</em> and <em>S. mutans</em> are highly similar cariogenic species and were classed together until the 1980s.</td>
</tr>
<tr>
<td>i</td>
<td>Twice daily, 5 days/week, plus once daily on weekends for 5 weeks</td>
</tr>
<tr>
<td>j</td>
<td>Murexide staining is well documented to reveal both gross and incipient lesions.</td>
</tr>
<tr>
<td>k</td>
<td>Hemisectioning (a single longitudinal bisection) or serial sectioning permits visualization of sulcal (fissure) and interproximal caries lesions.</td>
</tr>
<tr>
<td>l</td>
<td>Sucrose in drinking water increases the cariogenic challenge.</td>
</tr>
<tr>
<td>m</td>
<td>Addition of Mebromine permits enhanced visualization of lesions under UV light.</td>
</tr>
<tr>
<td>n</td>
<td>Shrestha transformation of Keyes caries scores combines frequency and severity aspects of the scoring system.</td>
</tr>
</tbody>
</table>
2. Key Elements of the Enamel Solubility Reduction

Acid demineralization of dental enamel is an important process in the formation of dental caries. It is well known that fluoride, when incorporated into enamel, makes it more resistant to demineralization by acids. Reducing the acid solubility of enamel is an important part of the anticaries properties of fluoride as it provides a protective function for the enamel during subsequent acid attack. Because of its importance in caries prevention, determining enamel solubility reduction is well accepted as a marker of the anticaries capability of a fluoride-containing dentifrice. The Final Monograph on OTC Anticaries Drug Products, [21 CFR §355.70] allows a test of enamel solubility reduction as one of two available in vitro methods required to supplement animal caries testing of fluoride dentifrice products.

**Principle and purpose:**

*To compare the ability of a test dentifrice and a clinically proven USP Reference Standard to reduce the solubility of enamel during acid challenge.*

A principal mechanism of fluoride’s anticaries activity is the reduction of enamel solubility during subsequent acid challenge. The remaining Key Elements set forth below provide a methodological framework for the determination of enamel solubility reduction by fluoride-containing dentifrices that incorporates best current practices and an inherent ability to maximize the use of modern enamel solubility reduction methodology.

March 12, 1999
Substrate

The substrate must be a suitable source of dental enamel mineral.

A suitable substrate must be used for determination of enamel solubility reduction. Substrates as diverse as intact or powdered enamel and hydroxyapatite discs were accepted in the testing procedures referenced in the Final Monograph for Anticaries Drug Products for Over-the-Counter Human Use.

The amount of substrate must be adequate to provide a measurable amount of the analyte indicative of enamel dissolution (e.g., calcium or phosphorous) in the pre- or post-treatment etches.

Sample size and controls:

The sample size must be adequate to meet the statistical requirements of the test.

Controls must include a clinically proven USP Reference Standard and an appropriate fluoride-free negative control.

The number of samples in each treatment group is determined by the individual model and must be adequate to meet the statistical requirements of the test. Controls used in each study must be the clinically proven USP Reference Standard, as a positive control, and a fluoride-free negative control. The positive control must contain the same anticaries active as the test product.
Preconditioning:

Preconditioning must minimize residual fluoride content and prepare the enamel mineral for fluoride incorporation, which is required to reduce enamel solubility during acid challenge.

Preconditioning is usually required to minimize residual fluoride in the substrate. When intact teeth are used, the preconditioning procedure may entail acid etching of the teeth or tooth sets for a time adequate to reduce residual fluoride. Other methods such as grinding or polishing may also be utilized to minimize residual surface fluoride. Powdering of enamel is considered a preconditioning step.

Hydroxyapatite disks generally do not require a preconditioning procedure, although preconditioning may be included if deemed appropriate for the individual model.

Treatment Regimen:

Treatment must provide a reproducible, controlled application of the dentifrice to the substrate. (Conditions controlled include dentifrice diluent/dilution and treatment time.)

The treatment regimen will vary depending on the individual study design.

Diluent: Dentifrice treatments can be in the form of a slurry in an appropriate medium such as distilled water or saliva. The treatment may be applied as either the slurry or supernatant.

March 12, 1999
Dilution: Dentifrice slurries are generally in the range of 50% (w/w) dentifrice/diluent or less.

Treatment conditions: Treatments will be done with adequate mixing to keep the dentifrice solids or substrate suspended and can be performed at temperatures up to 37°C. Following treatment, the enamel substrate will be thoroughly rinsed with distilled water.

Treatment time: Depending on the individual model, treatment time may vary from one to 60 minutes and may entail one or more exposures to the treating slurry or supernatant. Treatment times and frequencies will be selected to assure adequate sensitivity of the model.

Evaluation of Substrate:

The evaluation must provide a reproducible, controlled acid challenge to the substrate. Etch solutions must be quantitated for analytes indicative of enamel dissolution by valid analytical methods.

The method of evaluation will depend on the form of the substrate. Comparisons of treatments may be expressed as the amount of analyte released during an acid challenge after treatment, or the difference in analyte released after treatment relative to the amount released prior to treatment.
For example, when intact teeth are used, evaluation is performed by using a pre- and post-treatment acid etch. These etches can be done with agitation up to 37°C. Any acid source can be used, provided the strength and pH of the buffer is sufficient to release a measurable amount of analyte (e.g., calcium or phosphorus). The duration and conditions for the pre- and post-treatment etches must be identical and the actual time determined based on the requirements of the individual model. The specific conditions of the etch steps need to be such that measurable amounts of analyte (e.g., calcium or phosphorus) are released and significant differences between the placebo and standard dentifrices can be determined.

When enamel powder is chosen as a substrate, a pre-treatment etch is not usually practical. As above, the post-treatment etch can be done with agitation up to 37°C. The etch step is usually terminated by filtration. Any acid source can be used, provided the strength and pH of the buffer is sufficient to provide a measurable amount of analyte (e.g., calcium or phosphorus) in the filtrate. The duration of the etch must be appropriate for the particular method. The specific conditions of the etch step need to be such that the filtrate contains a measurable amount of analyte (e.g., calcium or phosphorus) and significant differences between the fluoride free dentifrice and clinically proven USP Reference Standard can be determined.

The acid etch solutions can be analyzed for any analyte that provides an indication of enamel dissolution, usually calcium,
phosphorus, or phosphate. Quantitation can be performed using any scientifically acceptable and validated method of analysis. Examples of acceptable quantitation methods include, but are not limited to, colorimetric, titration, atomic absorption, ion chromatography, and radioisotope counting methods.

**SUMMARY OF THE KEY ELEMENTS OF ENAMEL SOLUBILITY REDUCTION**

Contemporary protocols for enamel solubility reduction can be found in Appendix B (numbered 14-24). Each contemporary protocol meets the requirements of the Key Elements. These protocols have been successfully used for the evaluation of at least one marketed product.

Table 6 compares the Key Element range of parameters within the docket protocols (alone) and the contemporary protocols. Generally, the range of parameters within each Key Element are similar in the contemporary protocols and the docket protocols. While the Key Elements of anticaries testing have not changed, contemporary protocols reflect changing circumstances beyond the control of investigators and procedures that decrease variability or enhance sensitivity and reliability of the protocol.

Table 7 provides a detailed comparison of the range of parameters within the Key Elements for all of the contemporary protocols.

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10 For example, discontinued availability of reagents, instruments or rat strains.

11 For example, better sampling techniques and analytical methods that provide better resolution of the analytes.

March 12, 1999
used. Following the table is a comment on the experimental parameters of contemporary protocols that are outside the range of the docket protocols. All protocols encompass the Key Elements, and the enhancements do not affect the outcome of the test. In addition, each profile test must employ methods of statistical design and analysis, sufficient to assure that the experiment is valid (i.e., clinically proven USP Reference Standard is statistically significantly superior) and that the test product is both significantly superior to the fluoride-free negative control and not significantly lower in performance than the USP Reference Standard.
Table 6: Summary of the Key Elements and Range of Protocol Parameters: Enamel Solubility Reduction Test Method

**PRINCIPLE AND PURPOSE OF TEST METHOD:** To compare the ability of a test dentifrice and a clinically proven USP Reference Standard to reduce the solubility of enamel during acid challenge.

<table>
<thead>
<tr>
<th>Key Element Description</th>
<th>Substrate</th>
<th>Sample Size and Controls</th>
<th>Preconditioning</th>
<th>Treatment Regimen</th>
<th>Evaluation of Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Substrate</strong></td>
<td>Human enamel crowns, Hydroxyapatite discs, enamel chips or powdered enamel</td>
<td>1 to 4 or not stated</td>
<td>Lactic acid etch, water soak, acetic acid etch or powdering</td>
<td>Diluent: water</td>
<td>Acid Challenge: Pre- and post- or just post-treatment etches in lactate or acetate buffer for 8 to 60 minutes. Analysis: Assay for calcium or phosphorus by various analytical methods (e.g., colorimetric, atomic absorption)</td>
</tr>
<tr>
<td><strong>Preconditioning</strong></td>
<td></td>
<td></td>
<td>Preconditioning must minimize residual fluoride content and prepare the mineral for fluoride incorporation that is required to reduce enamel solubility during acid challenge.</td>
<td>Treatment must provide a reproducible, controlled application of the dentifrice to the substrate. Conditions controlled include dentifrice diluent/dilution and treatment time.</td>
<td>The evaluation of the substrate must provide a reproducible, controlled acid challenge to the substrate. Etch solutions must be quantitated for analytes indicative of enamel dissolution by valid analytical methods.</td>
</tr>
<tr>
<td><strong>Range of Key Elements</strong></td>
<td></td>
<td></td>
<td>Preconditioning must minimize residual fluoride content and prepare the mineral for fluoride incorporation that is required to reduce enamel solubility during acid challenge.</td>
<td>Treatment must provide a reproducible, controlled application of the dentifrice to the substrate. Conditions controlled include dentifrice diluent/dilution and treatment time.</td>
<td>The evaluation of the substrate must provide a reproducible, controlled acid challenge to the substrate. Etch solutions must be quantitated for analytes indicative of enamel dissolution by valid analytical methods.</td>
</tr>
<tr>
<td><strong>Treatment Regimen</strong></td>
<td></td>
<td></td>
<td>Treatment must provide a reproducible, controlled application of the dentifrice to the substrate. Conditions controlled include dentifrice diluent/dilution and treatment time.</td>
<td>Treatment Time: 1 to 60 minutes treatment time.</td>
<td></td>
</tr>
</tbody>
</table>
Table 7: Key Elements Parameters of Contemporary Protocols for Enamel Solubility Reduction Test Method

<table>
<thead>
<tr>
<th>Protocol Number</th>
<th>Substrate</th>
<th>Sample Size and Controls</th>
<th>Preconditioning</th>
<th>Treatment Regimen</th>
<th>Evaluation of Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 FDA # 33</td>
<td>Human molar crowns (6/set)</td>
<td>4</td>
<td>etch in lactate buffer</td>
<td>Dentifrice:water supernatant 1:3 6 minutes</td>
<td>Pre- and Post Treatment etches for 15 minutes at 37°C in lactate buffer. Phosphorus assay of pre and post-treatment etch solutions</td>
</tr>
<tr>
<td>15</td>
<td>Human molar crowns (3/set)</td>
<td>15°C</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>16</td>
<td>--</td>
<td>6</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>17</td>
<td>--</td>
<td>12</td>
<td>--</td>
<td>--</td>
<td>--</td>
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<tr>
<td>18</td>
<td>--</td>
<td>16</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>20 FDA # 34</td>
<td>Human molar crowns (4/set)</td>
<td>3</td>
<td>--</td>
<td>Dentifrice:water slurry 1:3 5 minutes</td>
<td>--</td>
</tr>
<tr>
<td>21 FDA # 36</td>
<td>Hydroxyapatite die or enamel mosaic</td>
<td>Not Stated</td>
<td>Before-treatment etch</td>
<td>Dentifrice:water slurry 1:3 1 minute</td>
<td>Post-treatment etch in acetate buffer for 8 minutes (chips) or 1 hour (discs) at 37°C Assay for Calcium by Atomic absorption</td>
</tr>
<tr>
<td>22 FDA # 36</td>
<td>Powdered human enamel</td>
<td>1</td>
<td>Powdering</td>
<td>Dentifrice:water supernatant 1:3 60 minutes</td>
<td>Post-treatment etch in acetate buffer for 45 minutes at 37°C Assay for phosphorus</td>
</tr>
<tr>
<td>23</td>
<td>--</td>
<td>3</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>24</td>
<td>--</td>
<td>--</td>
<td>--</td>
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</tr>
</tbody>
</table>

The use of a larger sample size is acceptable and will provide results at least as reliable as the docket protocols.

March 12, 1999
The use of saliva as a dentifrice diluent has been accepted in a docketed enamel fluoride uptake protocol and its use is also appropriate for this test.
3. **Key Elements of Enamel Fluoride Uptake**

Fluoride uptake assays are useful for determining whether the fluoride incorporated into a dentifrice is released during use and available to react with enamel. Fluoride uptake assays measure the amount of fluoride bound to the tooth. When fluoride binds to the tooth surface, it makes the tooth more resistant to acid attack. Thus, assays that are able to demonstrate the binding of fluoride either into the tooth or onto the enamel surface are useful for confirming product effectiveness. The FDA Final Monograph on OTC Anticaries Drug Products, [21 CFR §355.70] allows a test of enamel fluoride uptake as one of two available *in vitro* methods required to supplement animal caries testing of fluoride dentifrice products.

**Principle and purpose:**

The assessment of the ability of a fluoride-containing dentifrice to affect fluoride incorporation into demineralized enamel is well accepted as a means of demonstrating the anticaries potential of fluoride-containing dentifrice. The other *Key Elements* set forth below provide a methodological framework for the determination of fluoride uptake from fluoride-containing dentifrices that incorporates best current practices and an inherent ability to maximize the use of modern fluoride uptake methods.

March 12, 1999
Substrate:

The substrate must be a suitable source of dental enamel mineral.

A suitable substrate must be used for determination of enamel fluoride uptake. Substrates as diverse as intact or powdered enamel were accepted in the testing procedures referenced in the final monograph for anticaries drug products. Bovine enamel is similar in chemical composition to human enamel and is therefore a suitable substrate for the same procedure.

Sample size and controls:

Sample size must be adequate to meet the statistical requirements of the test.

Controls must include a clinically proven USP Reference Standard and an appropriate fluoride-free negative control.

The number of samples in each treatment group is determined by the individual model and must be adequate to meet the statistical requirements of the test. Controls used in each study must be the clinically proven USP Reference Standard of the same fluoride species as the test dentifrice, as a positive control, and a fluoride-free negative control.

Preconditioning:

Preconditioning must minimize residual surface fluoride and prepare the substrate for fluoride incorporation.
Preconditioning may be required to either 1) minimize residual fluoride in the substrate and/or 2) establish a condition suitable for measuring fluoride incorporation into the enamel. When intact substrates are used, the preconditioning may entail acid etching for a time adequate to reduce residual fluoride. Other methods such as grinding, polishing, or powdering may also be utilized to minimize residual surface fluoride. Intact substrates used for determining fluoride uptake usually are demineralized prior to treatment exposure.

The demineralization technique can be any method, which provides for a partial demineralization of the enamel, leaving the enamel softened, yet structurally intact. This condition is morphologically similar to the human caries condition. Demineralization solutions routinely consist of a buffered acid (such as lactic acid) and can be combined with an enamel surface protectant (such as methane-hydroxy-diphosphonate [MHDP], Carbopol, cellulose gum, etc.).

**Treatment Regimen:**

The treatment must provide reproducible conditions (diluent/dilution, time, and frequency) of application of dentifrice to substrate.

The treatment regimen will vary depending on the individual study design.

**Diluent:** An appropriate media, such as distilled water or saliva is used to dilute products for testing. The treatment may be applied as either the whole slurry or slurry supernatant.

March 12, 1999
Dilution: Dentifrice slurries are generally in the range of 50% (w/w) dentifrice:diluent or less.

Treatment Time and Frequency: Duration and number of treatment exposures over the course of the study are determined by particular study design, with the ultimate design based on the ability of the particular model to meet statistical criteria. If a single treatment study method is chosen, the treatment times may be of a longer duration than multiple treatment studies. pH cycling studies (greater number of treatments, shorter duration per treatment) incorporate both remineralization and demineralization phases over the course of an extended period of time.

Evaluation of Substrate:

The method of evaluation will depend on the form of the substrate. Comparisons of treatments may be expressed as the amount of fluoride incorporated into enamel over the course of treatments.

Fluoride sampling can be accomplished by any scientifically accepted method. For example, two such methods are the enamel biopsy (microdrill) technique and the acid etch technique. The resulting fluoride ion concentration can be analyzed using any
validated analytical method (e.g., ion selective electrode, chromatographic, and mass spectrometric analyses).

**SUMMARY OF THE KEY ELEMENTS OF ENAMEL FLUORIDE UPTAKE**

Contemporary protocols for enamel fluoride uptake can be found in Appendix C (numbered 26-38). Each contemporary protocol meets the requirements of the Key Elements. These protocols have been successfully used for the evaluation of at least one marketed product.

Table 8 compares the Key Elements range of parameters within the docket protocols (alone) and the contemporary protocols. Generally, the range of parameters within each Key Element is similar in the contemporary protocols and the docket protocols. While the Key Elements of anticaries testing have not changed over time, contemporary protocols reflect changing circumstances beyond the control of investigators and procedures that decrease variability or enhance sensitivity and reliability of the protocol.

Table 9 provides a detailed comparison of the range of parameters within the Key Elements for all of the contemporary protocols used. Following the table is a comment on experimental parameters of contemporary that are outside the range of the docket protocols. All protocols encompass within the Key Elements, and the enhancements do not effect the outcome of the test based on

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12 For example, discontinued availability of reagents, instruments or rat strains
13 For example, better sampling techniques and analytical methods that provide better resolution of the analytes.

March 12, 1999
results with a clinically proven USP Reference Standard. In addition, each profile test must employ methods of statistical design and analysis, sufficient to assure that the experiment is valid (i.e., clinically proven USP Reference Standard is statistically significantly superior to a negative control) and that the test product is both significantly superior to the fluoride-free negative control and not significantly lower in performance than the USP Reference Standard.
Table 8
Summary of Key Elements and Range of Protocol Parameters: Enamel Fluoride Uptake Test Method

**PRINCIPLE AND PURPOSE OF THE TEST METHOD:** To compare the ability of test dentifrice and a clinically proven USP Reference Standard to effect the fluoride incorporation into demineralized enamel

<table>
<thead>
<tr>
<th>Key Element and Parameters</th>
<th>Substrate</th>
<th>Sample Size and Controls</th>
<th>Preconditioning of Samples</th>
<th>Treatment Regimen</th>
<th>Evaluation of Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Key Element Description</strong></td>
<td>The substrate must be a suitable source of dental enamel mineral.</td>
<td>The sample size must be adequate size to meet the statistical requirements of the test.</td>
<td>Preconditioning must minimize residual surface fluoride and prepare for fluoride incorporation.</td>
<td>Treatment must provide reproducible conditions (dentin, dilution, time and frequency) for application of dentifrice to substrate.</td>
<td>The evaluation of the substrate must use quantitative controlled sampling and valid chemical analyses to allow for comparisons of treatments.</td>
</tr>
<tr>
<td><strong>Range of Key Elements Parameters within Docket Protocols</strong></td>
<td>Intact or powdered human enamel</td>
<td>3 - 20/group or not stated</td>
<td>Pretreatment: Etch, grind, polish, powder, 0.5N - 2N HClO₄.</td>
<td>Diluent: Human or synthetic saliva, water, slurry or supernatant.</td>
<td>Sampling: Acid etch or dissolve in acid. Analysis: Fluoride electrode; colorimeter or atomic absorption.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Demin: 0.025M lactic acid, MHDP, perchloric acid if applicable.</td>
<td>Dilution: not stated - 1:3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Time/Frequency: 15 - 60 minutes</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Range of Key Elements Parameters within Contemporary Protocols (including Docket Protocols)</strong></td>
<td>Intact or powdered human enamel or intact bovine enamel</td>
<td>3 - 20/group or not stated</td>
<td>Pretreatment: Etch, grind, polish, powder, 0.5N - 2.0N HClO₄.</td>
<td>Diluent: Human or synthetic saliva, water, slurry or supernatant.</td>
<td>Sampling: Acid etch or dissolve in acid. Analysis: Fluoride electrode; colorimeter or atomic absorption.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Demin: 0.025M - 0.1M lactic acid + MHDP, Carbopol, or 0.5N HCIO₄.</td>
<td>Dilution: Not stated - 1:2 - 1:4.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Dilution: Acid etch or dissolve in acid, microdrill. Analysis: Fluoride electrode; colorimeter or atomic absorption.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 9: Summary of Key Element Parameters of Contemporary Protocols for Enamel Fluoride Uptake Method

<table>
<thead>
<tr>
<th>Protocol Number</th>
<th>Substrate</th>
<th>Sample Size and Controls</th>
<th>Preconditioning of Samples</th>
<th>Key Element Parameters</th>
<th>Treatment Regimen</th>
<th>Evaluation of Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDA #40</td>
<td>Human Enamel (intact)</td>
<td>not stated</td>
<td>Etch, 2M HClO₄ 0.025M lactic acid + 0.0002M HMFP</td>
<td>Diluent Pooled human saliva or water supernatant</td>
<td>Dilution 1:3</td>
<td>Time/ Frequency 30 minutes Acid etch Fluoride electrode</td>
</tr>
<tr>
<td>26</td>
<td>&quot;</td>
<td>&quot;</td>
<td>Etch, 1M HClO₄ &quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>27</td>
<td>&quot;</td>
<td>&quot;</td>
<td>Grind, polish &quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>28</td>
<td>&quot;</td>
<td>10/group</td>
<td>Etch, 2M HClO₄ 0.025M lactic acid + 0.2% Carbopol</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>29</td>
<td>&quot;</td>
<td>Not stated</td>
<td>Grind, polish 0.025M Lactic Acid 0.0002M HMFP</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>30</td>
<td>&quot;</td>
<td>Etch, 1M HClO₄</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>31</td>
<td>Bovine enamel</td>
<td>10/group</td>
<td>Grind, polish 0.05M lactic acid . 50% saturated w/ HAP, 0.1% Carbopol</td>
<td>Distilled water Slurry</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>32</td>
<td>Human enamel (intact)</td>
<td>4/group</td>
<td>0.1M lactic acid + 0.2% Carbopol 60% saturated w/ HAP</td>
<td>Pooled human saliva Slurry</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>33</td>
<td>&quot;</td>
<td>18/group</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>34</td>
<td>Human Enamel (intact)</td>
<td>8/group</td>
<td>Grind, polish, 0.5M HClO₄ etch 0.5M HClO₄ etch</td>
<td>Deionized water Slurry</td>
<td>1:3</td>
<td>15 minutes Acid etch Fluoride electrode/ Colorimeter (PO₄), Atomic absorption (Ca)</td>
</tr>
<tr>
<td>FDA #41</td>
<td>&quot;</td>
<td>15/group</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>FDA #42</td>
<td>Human Enamel (powdered)</td>
<td>not stated</td>
<td>Powdering Not Applicable</td>
<td>Synthetic saliva supernatant</td>
<td>not stated</td>
<td>Not stated Dissolve in acid Fluoride electrode</td>
</tr>
<tr>
<td>37</td>
<td>Human Enamel (powdered)</td>
<td>3/group</td>
<td>Powdering Not Applicable</td>
<td>Distilled water Supernatant</td>
<td>1:3</td>
<td>1 hour Dissolve in acid Fluoride Electrode</td>
</tr>
</tbody>
</table>

March 12, 1999 page 54
Key for Notes in Table 9

a  Each protocols meets the requirements of the key elements as described in Table 8. These protocols have been successfully used for evaluation of at least one marketed product.

b  Controls are the same for each experiment and must include a clinically proven USP Reference Standard and an appropriate fluoride free negative control.

c  The solutions listed in the contemporary protocols represent the current state of lesion formation chemistry. The type of lesions formed with the various media have all demonstrated reactivity to fluoride.

d  Total exposure time (90 minutes) is in line with previous maximum times used (60 minutes). pH cycling protocols utilize multiple exposure to short time treatments rather than prolonged exposure to single treatment. pH cycling models are well accepted models in the research community.


f  Bovine enamel has been demonstrated to provide results comparable to human enamel.
Tab VIII

Perspective of the Experts in the Field of Anticaries Testing
The CHPA/CTFA Joint Oral Care Task Group has conducted a broad survey of dental research scientists who are active in employing biological test methods for fluoride dentifrice final formulation testing, as well as investigators who have made significant contributions to identifying some of the mechanisms by which fluoride exerts its anticaries activity. The survey encompassed a dozen researchers, located in various geographic regions of the US and Europe. We asked these experts to comment on the biological test methods described in the docket, and the Key Elements of the three test methods.

The broad consensus of these researchers is that the definitive mechanism of fluoride anticaries activity is still not fully defined, and that there is a need for additional effort to improve methods that model fluoride’s action. However, these scientists agree that the biological testing methods for establishing the effectiveness of a fluoride dentifrice, as defined in the docket, measure the behavioral properties of fluoride which are believed to be predictors of its anticaries activity.

- The Animal Caries Reduction Test measures the cariostatic and anti-cariogenic properties of fluoride in vivo.
- The Enamel Solubility Reduction Test measures the ability of fluoride to mediate, reduce or diminish the loss of calcium and phosphate from the crystal lattice of enamel during an acid challenge.
- The Enamel Fluoride Uptake Test measures the ability of fluoride to penetrate fluoride-depleted enamel for potential incorporation into deficient crystal surfaces.
The scientific community further agrees that the interaction of fluoride with biological substrates demonstrates these mechanisms of action and is dependent upon fluoride availability. It is generally recognized that an effective dentifrice must provide fluoride in an available form. The biological test methods in the monograph serve this purpose. Thus, while performance of a product in a biological test measures the behavioral property of fluoride, the primary function of the biological test is to confirm the availability and delivery of fluoride from a dentifrice.

Many of these scientists are associated with dental research institutions of high academic standing and have published and presented at international symposia, workshops and scientific forums. Their experience ranges from 20 to 40 years and all have extensive knowledge in one or more aspects of testing the anticaries properties of fluoride. Table 10 lists these investigators.
Table 10  
Dental Researchers Who Have Reviewed the Key Elements

<table>
<thead>
<tr>
<th>INVESTIGATOR</th>
<th>POSITION</th>
<th>AFFILIATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>William H, Bowen, Ph.D.</td>
<td>Margaret and Cy Welcher Professor of Dentistry and Professor of Microbiology and Immunology</td>
<td>University of Rochester</td>
</tr>
<tr>
<td>Bernhard Guggenheim, Ph.D.</td>
<td>Professor and Head Department of Oral Microbiology and General Immunology</td>
<td>Institute for Oral Microbiology and General Immunology, Zurich</td>
</tr>
<tr>
<td>Geoffrey Ingram, Ph.D.</td>
<td>Honorary Lecturer</td>
<td>University of Liverpool</td>
</tr>
<tr>
<td>Carl Kleber, Ph.D.</td>
<td>Associate Director and Research Scientist</td>
<td>Indiana University-Purdue University</td>
</tr>
<tr>
<td>James Mellberg</td>
<td>Private Consultant</td>
<td>Formerly with Colgate-Palmolive Company</td>
</tr>
<tr>
<td>Mark Putt, Ph.D.</td>
<td>Director and Research Scientist</td>
<td>Indiana University/Purdue University</td>
</tr>
<tr>
<td>Bruce Schemehorn</td>
<td>Director, Contract Research</td>
<td>Indiana University Oral Health Research Institute</td>
</tr>
<tr>
<td>George Stookey, Ph.D.</td>
<td>Associate Dean for Academic Affairs; Professor of Preventive and Community Dentistry; and Director, Oral Health Research Institute</td>
<td>Indiana University Oral Health Research Institute</td>
</tr>
<tr>
<td>Jason M. Tanzer, D.M.D., Ph.D.</td>
<td>Professor and Head, Division of Oral Medicine, Department of Oral Diagnosis</td>
<td>University of Connecticut</td>
</tr>
<tr>
<td>Janice Warrick</td>
<td>Director, Bioresearch Facility</td>
<td>Indiana University Oral Health Research Institute</td>
</tr>
<tr>
<td>Don White, Ph.D.</td>
<td>Principal Research Scientist</td>
<td>The Procter &amp; Gamble Company</td>
</tr>
<tr>
<td>Yiming Li, Ph.D.</td>
<td>Professor, Department of Microbiology and Molecular Genetics</td>
<td>Loma Linda University</td>
</tr>
</tbody>
</table>

March 12, 1999
Our survey has shown that it is the consensus of the scientists we asked that:

1. The **Key Elements** define the essentials of the biological test methods.
2. The contemporary protocols employed in their laboratories are consistent with the **Key Elements**; and
3. These protocols will not influence the test outcome in a false or misleading manner and are at least as good as the biological test methods in the docket for assessing the biological availability of fluoride in a dentifrice.

It is logical to conclude that defined acceptance criteria linked with appropriate statistical design will ensure that the contemporary protocols consistent with the **Key Elements** will not produce outcomes of a false or misleading nature.

March 12, 1999
Tab IX

Summary

March 12, 1999
Discussion Topic C: Contemporary protocols, consistent with the Key Elements, are compliant procedures for final formulation testing

Three principles, to assure the effectiveness of OTC fluoride dentifrices, exist today to make the Anticaries Monograph a practical working document:

1. The physical stability and chemical parameters of the dentifrice must initially match established specifications.

2. The available fluoride of the product must match a given profile over the shelf-life of the product.

3. The fluoride dentifrice must meet the biological testing requirements of the monograph using clinically proven USP Reference Standard as the internal control.

Biological testing of fluoride dentifrices is only one principle of establishing adequately formulated dentifrice products under the monograph. These biological test methods have not changed; however, there has been an evolution of the protocols since their original submission. The Key Elements, defined by the original 1978 protocols for each test method, are considered essential for properly designed protocols. Consequently, these Key Elements provide a methodological framework for the determination of animal caries reduction, enamel solubility reduction, and enamel fluoride uptake testing. Furthermore, this framework allows for the incorporation of best current practices and allows for an inherent ability to maximize the use of modern scientific methods to ensure the effectiveness of anticaries products.

The CHPA/CTFA Joint Oral Care Task Group believes that it is prudent to expand the boundaries of existing protocols in the docket to include protocols which reflect current scientific practices, but do not deviate from the fundamental
principles defined by the protocols submitted during the rulemaking process. The **Key Elements** of each test method provide a framework for evaluating protocols to assure that enhancements are consistent with the fundamental principles of the test methods.

In summary, we believe that:

A. The **Key Elements** of a test method describe the critical aspects of generally accepted fluoride dentifrice biological testing as found in the Anticaries Docket.

B. The contemporary protocols, not listed in the docket, but utilized for anticaries testing, are consistent with the **Key Elements**.

C. The contemporary protocols, consistent with the **Key Elements**, are compliant procedures for final formulation testing.

**ACTION REQUESTED:**

1. We request that the agency acknowledge in writing that the contemporary protocols, consistent with the **Key Elements**, are compliant procedures for final formulation testing of anticaries drug products.

2. We request that the agency work with industry to develop a simplified process for recognizing that requirements for biological testing can be met with future state-of-the-art protocols consistent with the **Key Elements**.

March 12, 1999
Study Number AC655A: Rat Caries Evaluation of USP Standards Using the 5% Sucrose Model, Procter & Gamble Company Health Care Research Center, P.O. Box 8006, Mason, Ohio 45040-8006.

**Summary**
The purpose of this study was to assess the ability of a low sucrose model (5% sucrose) to detect significant differences between a non-fluoride (placebo) dentifrice and the four USP Reference Standard Dentifrices commonly used for FDA required dentifrice testing. The basic protocol, following the Essential Key Elements, was as follows:

- **Animal (substrate):** Wistar rats (22-23 days old) at study initiation
- **Sample Size and Controls:** each test cell contained 20 animals
- **Preconditioning:**
  - **Water Source/Cariogenic Diet:** animals were maintained on a cariogenic diet -- Modified 469 with 5% sucrose) and provided deionized H₂O ad libitum
  - **Cariogenic Microflora:** resident microflora verified to be cariogenic
- **Treatment Regimen:**
  - **Schedule:** twice daily for three weeks
  - **Applicator:** cotton swab
  - **Dilution of Dentifrice:** 1:1 with deionized water
- **Evaluation of Substrate:**
  - **Staining/Sectioning:** jaws were hemi-sectioned and stained with silver nitrate stain
  - **Scoring System/Endpoint:** all animals were sacrificed and scored for caries using the Briner-Francis method

The five test groups were comprised of a fluoride free negative control (placebo/silica), NaF/silica, SMFP/dicalcium phosphate dihydrate (Dical), SMFP/silica, and SnF₂/silica. Total caries scores for the five groups were:

<table>
<thead>
<tr>
<th>USP Reference Standard</th>
<th>Total Caries Score</th>
<th>% Reduction (relative to placebo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaF/silica</td>
<td>62.6</td>
<td>51</td>
</tr>
<tr>
<td>SnF₂/silica</td>
<td>71.7</td>
<td>44</td>
</tr>
<tr>
<td>SMFP/silica</td>
<td>89.6</td>
<td>30</td>
</tr>
<tr>
<td>SMFP/dical</td>
<td>96.1</td>
<td>25</td>
</tr>
<tr>
<td>Placebo (non-fluoride)*</td>
<td>128.0</td>
<td></td>
</tr>
</tbody>
</table>

*placebo dentifrice is not a USP Reference Standard.

The results confirm the ability of this 5% sucrose model to statistically separate each of the four USP Reference Standard dentifrices from the fluoride free, placebo control.
INTERDEPARTMENTAL CORRESPONDENCE

FROM: H. M. Pickrum, Study Director  
      J. M. Best, Study Associate  
      HCRC, 8700 Mason-Montgomery Rd.  
      Mason, OH 45040

DATE: June 7, 1999  
R/L: Non-Discretionary

TO: File Report

STUDY DATES: 04/14/99-05/06/99

SUBJECT RAT CARIES EVALUATION NOTEBOOK#: HCL3435

OF USP STANDARDS USING THE 5% SUCROSE MODEL

STUDY #: AC#655A

SUMMARY

The purpose of this rat caries study is to evaluate the response to treatment using the 5% sucrose rat caries model when evaluating the USP Standards. The USP standards tested were a NaF/silica dentifrice, a SnF₂/silica dentifrice, a SMFP/dical dentifrice and a SMFP/silica dentifrice.

Results from this caries study show all USP Standard dentifrices tested were significantly different from the Placebo dentifrice. The USP NaF/silica standard dentifrice and the USP SnF₂/silica dentifrice were equivalent and significantly more effective than the USP SMFP/dical standard dentifrice and the SMFP/silica standard dentifrice. The USP SMFP/dical standard dentifrice and the USP SMFP/silica standard dentifrice were equal in anticaries activity.

The results from this study demonstrate that the 5% sucrose diet rat caries model can be used to evaluate products containing fluoride.

BACKGROUND AND OBJECTIVE

The purpose of this rat caries study is to evaluate the response to treatment using the 5% sucrose rat caries model when evaluating the USP Standards. The USP standards tested were a NaF/silica dentifrice, a SnF₂/silica dentifrice, a SMFP/dical dentifrice and a SMFP/silica dentifrice.

Previous rat caries data had demonstrated the 5% sucrose model could be used to evaluate products containing fluoride with results similar to the 63% sucrose model.
MATERIALS AND METHODS:

REFER TO: SOP - RO#1 (similar to FDA Method #37 except diet sucrose levels)

Experimental Design

Type of Study: Rat Caries

Species (Strain): Harlan Wistar Albino Rats

Source (Supplier): Harlan Sprague-Dawley Inc.

Sex: Random (See Notebook #HCL3435)

Initial Weight: (See Notebook #HCL3435)

Number of Animals
Per Group: 20 animals

Means of Animal Identification: Cage Tag

Diet: 5% Sucrose (see Attachment I diet composition)

Housing: Singe, stainless steel suspended wire cage

Test Substance:

<table>
<thead>
<tr>
<th>Group</th>
<th>Test Substance</th>
<th>Code #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>USP Standard SnF₂/silica dentifrice</td>
<td>PTG 07-41</td>
</tr>
<tr>
<td>Group 2</td>
<td>USP Standard SMFP/dical dentifrice</td>
<td>#041223</td>
</tr>
<tr>
<td>Group 3</td>
<td>USP Standard SMFP/silica dentifrice</td>
<td>PTG 07-40</td>
</tr>
<tr>
<td>Group 4</td>
<td>USP Standard NaF/silica dentifrice</td>
<td>DB 730784</td>
</tr>
<tr>
<td>Group 5</td>
<td>Placebo (0 ppm F)</td>
<td>HCS 223-14</td>
</tr>
</tbody>
</table>

Route of Exposure: Topical (maxillary and mandibular molars)

Carrier Solvent: Deionized water diluted 1:1 (w/v) with treatment

Code #
Dose and Treatment

Duration: All treatments were administered twice daily for three weeks with the exception of weekends. Treatments were brushed on with cotton-tipped applicator sticks.

Analysis: Standard analysis of variance with treatments ranked by Duncan's Multiple Range Test

RESULTS:

<table>
<thead>
<tr>
<th>GROUP NUMBER</th>
<th>TREATMENTS</th>
<th>X CARIES SCORE / RAT</th>
<th>PERCENT REDUCTION</th>
<th><em>n</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>USP NaF/silica Standard</td>
<td>62.55</td>
<td>51</td>
<td>20</td>
</tr>
<tr>
<td>1</td>
<td>USP SnF₂/silica Standard</td>
<td>71.70</td>
<td></td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>USP SMFP/silica Standard</td>
<td>89.60</td>
<td>30</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>USP SMFP/dical Standard</td>
<td>96.1</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>Placebo (0 ppm F)</td>
<td>127.95</td>
<td>--</td>
<td>20</td>
</tr>
</tbody>
</table>

Treatment means within brackets are significantly different from those outside at $\alpha = 0.05$.

*The mortality standard for dental testing is no more than 5% per test and/or no more than 10% per group.

The Placebo treatment was significantly different from all other treatment groups.

The USP NaF/silica dentifrice and the USP SnF₂/silica dentifrice were not significantly different from each other but both were significantly different from all other treatment groups.

The USP SMFP/silica dentifrice and the USP SMFP/dical dentifrice were not significantly different from each other but both were significantly different from all other treatment groups.
DISCUSSION:

Results from this caries study show all USP Standard dentifrices tested were significantly different from the Placebo dentifrice. The USP NaF/silica standard dentifrice and the USP SnF₂/silica dentifrice were equivalent and significantly more effective than the USP SMFP/dical standard dentifrice and the SMFP/silica standard dentifrice. The USP SMFP/dical standard dentifrice and the USP SMFP/silica standard dentifrice were equal in anticaries activity.

The results from this study demonstrate that the 5% sucrose diet rat caries model can be used to evaluate products containing fluoride.

Specimens, raw data, and final report are stored at the Health Care Research Center, Mason, Ohio.

PREPARED BY:

[Signature]
Study Associate

Date: 6/18/99

REVIEWED AND APPROVED BY:

[Signature]
Study Director

Date: 6/18/99
**ATTACHMENT 1**

**5% Diet Composition**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confectioners Powdered Sucrose</td>
<td>5</td>
</tr>
<tr>
<td>Milk Powder(non-fat dry)</td>
<td>32</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>58</td>
</tr>
<tr>
<td>Desiccated liver powder</td>
<td>2</td>
</tr>
<tr>
<td>Cellulose</td>
<td>3</td>
</tr>
</tbody>
</table>

Supplied by Harlan/TEKLAD  #TD 97359
Study Number 1221A: Effect of Experimental Dentifrice on Caries Formation in the Rat, Indiana University School of Dentistry Bio-research Facility, 1121 W. Michigan Street, Indianapolis, Indiana 46202-5186.

Summary
The purpose of this study was to assess the ability of the Indiana University rat caries model (67% sucrose) to detect significant differences between a non-fluoride (placebo) dentifrice and the four USP Reference Standard Dentifrices commonly used for FDA required dentifrice testing. The basic protocol, following the Essential Key Elements, was as follows:

- **Animal (substrate):** Sprague Dawley rats (23 days old) at study initiation
- **Sample Size and Controls:** each test cell contained 40 animals
- **Preconditioning:**
  - **Water Source/Cariogenic Diet:** animals were maintained on a cariogenic diet (MIT 200 with 67% sucrose) and provided deionized H₂O ad libitum
  - **Cariogenic Microflora:** animals were inoculated with a cariogenic strain of *Streptococcus sobrinus*
- **Treatment Regimen:**
  - **Schedule:** twice daily (once on weekends) for three weeks
  - **Applicator:** cotton swab
  - **Dilution of Dentifrice:** 1:1 with deionized water
- **Evaluation of Substrate:**
  - **Staining/Sectioning:** jaws were hemi-sectioned and stained with murexide stain
  - **Scoring System/Endpoint:** all animals were sacrificed and scored for caries using the Keyes method

The five test groups were comprised of a fluoride free negative control (placebo/silica), NaF/silica, SMFP/dicalcium phosphate dihydrate (Dical), SMFP/silica, and SnF₂/silica. Total caries scores for the five groups were:

<table>
<thead>
<tr>
<th>USP Reference Standard</th>
<th>Total Caries Score</th>
<th>% Reduction (relative to placebo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaF/silica</td>
<td>17.85</td>
<td>45</td>
</tr>
<tr>
<td>SnF₂/silica</td>
<td>18.78</td>
<td>43</td>
</tr>
<tr>
<td>SMFP/dical</td>
<td>22.08</td>
<td>33</td>
</tr>
<tr>
<td>SMFP/silica</td>
<td>22.85</td>
<td>30</td>
</tr>
<tr>
<td>Placebo (non-fluoride)*</td>
<td>32.75</td>
<td>---</td>
</tr>
</tbody>
</table>

* placebo dentifrice is not a USP Reference Standard.

The results confirm the ability of the Indiana University rat caries model (67% sucrose) to statistically separate each of the four USP Reference Standard dentifrices from the fluoride free, placebo control.
Title
Effect of Experimental Dentifrice on Caries Formation in the Rat

Study Number
Oral Health Research Institute Number 1221A
IACUC Number DS0000656R

Study Sponsor
The Procter & Gamble Company
Health Care Research Center
8700 Mason-Montgomery Road
P.O. Box 8006
Mason, OH 45040-8006
Attention: Mike Best

Study Site
Indiana University School of Dentistry
Bioresearch Facility
1121 W. Michigan Street
Indianapolis, IN 46202-5186

Conducting Agency
Indiana University School of Dentistry
Oral Health Research Institute
415 Lansing Street
Indianapolis, Indiana 46202-2876

Purpose
The purpose of this study was to assess the ability of the I.U. rat caries model to detect significant differences between a placebo dentifrice and the four USP dentifrice standards used for FDA dentifrice protocol testing. The five groups were comprised of a negative control, NaF/Silica, NaF MFP/Dicalcium Phosphate, NaMFP 1000 ppm/Silica and Stannous Fluoride Silica.

Test Substances
The test substances were five coded products supplied by the Sponsor. To perform this study, 500 grams of each dentifrice were required. The sponsor was responsible for the necessary evaluation related to the composition, purity, strength, stability, storage requirements, expiration dates and any other applicable requirements.

Test Design
The test design was similar to FDA Method #37. The major variations were the diet used (MIT 200 rather than #469), the caries scoring method (Keyes method rather than HMA; see Appendix A) and treatment frequency. Experimental procedures were conducted according to the FDA regulations Part 58.

<table>
<thead>
<tr>
<th>Group</th>
<th>Code</th>
<th>Treatment*</th>
<th>Diet</th>
<th>Treatment Frequency**</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>IU 204-1</td>
<td>NaF/Silica</td>
<td>MIT 200</td>
<td>Twice Daily</td>
</tr>
<tr>
<td>B</td>
<td>IU 204-2</td>
<td>SNF/Silica</td>
<td>MIT 200</td>
<td>Twice Daily</td>
</tr>
<tr>
<td>C</td>
<td>IU 204-3</td>
<td>SMFP/Dical</td>
<td>MIT 200</td>
<td>Twice Daily</td>
</tr>
<tr>
<td>D</td>
<td>IU 204-4</td>
<td>SMFP/Silica</td>
<td>MIT 200</td>
<td>Twice Daily</td>
</tr>
<tr>
<td>E</td>
<td>IU 204-5</td>
<td>Placbo</td>
<td>MIT 200</td>
<td>Twice Daily</td>
</tr>
</tbody>
</table>

* Test products were given a code by the Sponsor and decoded upon completion of data analysis.
** Treatments were administered seven days per week, with a single daily treatment on weekends.
Final Report: Study Number 1221A

Justification for Animal Use

For a variety of reasons governmental and professional review agencies have agreed to accept a battery of pre-clinical tests as a means of documenting the caries-preventive potential of certain types of fluoridated dentifrices in lieu of long-term clinical trials in children. This battery of tests includes the use of a rat caries model with a minimal test design consisting of a negative control (placebo dentifrice) group, a positive control group involving the use of a similar fluoride dentifrice whose caries-preventive benefits have been demonstrated in a controlled clinical trial, and an experimental group similarly treated with the experimental fluoride dentifrice.

Using litters as a covariate, the use of between 50 and 58 (depending on type of fluoride) animals per treatment group satisfies the most stringent power requirements of the ADA’s Council on Dental Therapeutics 20% clinical difference between treatments at 80% power. However, we have been routinely using 40 animals per treatment group and these tests have consistently been accepted by both the ADA CDT and the FDA. This required initiating the study with 40 animals per group. These animals were provided by 43 litters.

IACUC Approval

The protocol was reviewed and approved by the Institutional Animal Care and Use Committee prior to receipt of the animals.

Animals

1. Type of Animals
   Weanling mixed-sex Sprague Dawley rats; approximately 40-50 grams at study initiation.

2. Source
   Harlan Sprague Dawley, Inc.
   P.O. Box 29176
   Indianapolis, Indiana 46229

3. Housing
   The litters were maintained in large solid-bottom (box-type) cages with dams until the pups were weaned at 18 days of age. Starting at 9 days of pup age, the dams were rotated daily among the litters. The pups were maintained in the box cages until 21 days of age. At this time the pups were stratified and housed in pairs in suspended wire-bottomed cages which had been cleaned and sanitized prior to usage. The cages were arranged so that all animals of the individual groups were together and the cages were labeled with group designation and treatment (treatment code) that the animals received.
Final Report: Study Number 1221A

4. Identification and Stratification
When the animals were 21 days old they were given unique numbers by ear-punch with records kept of littermates. Animals were assigned to groups in such a manner that groups were balanced for litter weight and sex. There were 40 animals per group.

Animal Care
1. Diet
Upon receipt, dams and litters were provided rodent lab diet until the pups were 8 days of age. On day 8 (pup age) dams and litters were provided Diet MIT 305 (composition in Appendix B). Animals were provided diet MIT 200 (Composition in Appendix C) ad libitum at day 18 (pup age) and throughout the test period.

2. Water
All animals were provided with deionized water ad libitum.

3. Care
Box caging was changed at day 13 and again at day 18 of pup age. Cage board was changed three times a week at the time when fresh food and water were given (Monday, Wednesday and Friday). Clean and sanitized water bottles and food jars were provided weekly. Suspended caging and bank were sanitized bi-weekly. The animals were observed daily by a staff member and weekly by the attending veterinarian for any signs of health problems.

4. Room Environment
The animals were housed in an AAALAC-accredited facility. Room temperatures were maintained at 72°F (±6°F) with 10-15 air changes per hour and a 12-hour light cycle.

Inoculation
On day 15, the pups received an oral inoculation of streptomycin-resistant <i>S. sobrinus</i> 6715 culture (Appendix D). This involved flooding the mouth with 0.2 ml of culture/animal. On day 18 (pup age) the animals were provided Diet MIT 200 and were inoculated with <i>S. sobrinus</i> for three consecutive days (day 18, 19 and 20). This involved placing 0.1 ml of the <i>S. sobrinus</i> culture on the occlusal surfaces of each of the mandibular molar quadrants, putting 10 cc of this concentration-adjusted culture into each water bottle and lightly spraying the bedding with remaining culture. All water bottles were removed and sanitized 2 hours after inoculum had been added. The inoculum was administered to the animals with a 20 micropipetter.
Experimental Treatment Initiation

The treatment phase began at day 22 of pup age.

Experimental Procedures

1. Preparation and Labeling
   Each treatment had a labeled plastic beaker which was designated for that treatment only. Fresh materials (i.e., obtained from stock supply) were used for each treatment. The dentifrices were mixed in a 1:1 ratio (by weight) with deionized water. Specifically, 10 grams of dentifrice were weighed into a 30 ml beaker; 10 grams of deionized water were then weighed and added to the dentifrice. The mixture was then stirred by hand (30 seconds) with a clean microspatula for the purpose of creating a smooth mixture. The beaker containing the slurry and a small magnetic stirring bar was placed on a magnetic stirrer which was set at the lowest speed and allowed to stir for 3 minutes. The slurry was prepared immediately prior to each treatment.

2. Treatment Procedure
   A cotton-tipped applicator was dipped into the slurry (for 2 seconds) and was applied to one-half of the rat's mouth in such a way that the sides of the applicator came into contact with both the mandibular and maxillary molars on one side of the mouth. The treatment was accomplished by using a rolling motion of the sides of the applicator over the mandibular and maxillary molar teeth for 15 seconds. The applicator was dipped into the slurry for the second time (again, for 2 seconds) and the other side of the rat's mouth similarly treated for 15 seconds. A new applicator was used for each animal.

3. Schedule for Treatment Applications
   Treatments were administered twice daily for five days with a single daily treatment on weekends. The first treatment each day began at approximately the same time every day, and the second treatment began no earlier than six hours after the first treatment. Singular treatments were given at a 24 hour interval on weekends.

4. Storage of Material
   Treatment materials were stored at room temperature. All treatment products were returned to the sponsor at the study completion.

5. S. sobrinus Recovery
   One week after the initiation of the inoculation regimen and at study termination, an oral swabbing was taken from each rat using a sterile cotton swab (six-inch, single-tipped applicator). The microorganisms on the mandibular and maxillary molar teeth were sampled, using a rolling motion.
the swab for 15 seconds on one side of the mouth, rolled over the tongue, and rolled over the molar
teeth on the other side of the mouth for an additional 15 seconds.

Immediately after the applicator was removed from the animal's mouth, it was streaked across half of a
100 mm petri plate containing Mitis Salivarius agar to which 200 units/ml of streptomycin sulfate had
been added. The plates were incubated for 48 hours at 37°C with 10% CO₂. The colony count taken
after the 48 hours of incubation was recorded in the logbook.

Experimental Duration of Study
The duration of the experimental phase was three weeks.

Termination of Animal Phase
1. Final Observation and Examination
   Immediately prior to termination all animals were observed for any visual signs of ill health or
   pathology, individually weighed and an oral swabbing taken to confirm S. sobrinus implantation.

2. Euthanization of Animals and Post-Mortem Procedures
   The animals were euthanized by carbon dioxide inhalation. Code numbers were assigned to each
   animal and the heads were removed, placed in individual jars along with the code number, and
   prepared under pressure (10 PSI for 12 minutes). The hemijaws were then removed and freed of all
   soft tissue.

Study Completion
1. Tissue Preparation
   The cleaned hemijaws (four quadrants) were put into plastic vials with the code numbers taped to the
   vial. A murexide solution (0.3 g murexide; 300 ml DI H₂O and 700 ml of ethanol) was added to each vial
   and the jaws were allowed to stain overnight. The jaws were then rinsed and allowed to air dry.

2. Tissue Evaluation
   The hemijaws were microscopically examined for smooth surface caries, sectioned, and then
   microscopically examined for sulcal and interproximal caries using the Keyes method as outlined in
   Appendix A.

3. Data Processing and Analysis
   Statistical analyses were performed using the Bartlett-Box F and the Cochran's C tests for homogeneity
   of variance (at α=0.05). In cases where the variances were homogeneous, a one-way analysis of
   variance was performed. In cases in which homogeneity of variance could not be assumed, a
logarithmic or square root transformation of the data was made according to the relationship between
group means and variances, and transformed data reanalyzed. In cases where a significant "F" value
was found, Tukey's HSD test and/or Duncan's multiple range test were used to test for significant
differences between the individual means. For extreme variance heterogeneity, the nonparametric
Kruskal-Wallis one-way analysis of variance was used.

4. The specific types of data which were tabulated, statistically analyzed, and reported for each group are
as follows:

a. Mortality Data Experimental Phase
   - Initial number of animals
   - Final number of animals
   - Percent mortality

b. Growth Data Experimental Phase
   - Initial body weight (mean ± S.E.M.)
   - Final body weight (mean ± S.E.M.)
   - Body weight gain (mean ± S.E.M.)

c. Caries Data
   - Enamel and dentinal involvement of smooth surface lesions (mean ± S.E.M.).
   - Enamel and dentinal involvement of interproximal lesions (mean ± S.E.M.).
   - Enamel and dentinal involvement of sulcal lesions (mean ± S.E.M.).
   - Total caries involvement combining the scores from the Keyes method of scoring smooth surface,
     interproximal, and sulcal caries (mean ± S.E.M.).

d. S. sobrinus Status
   - Percent of animals and level of infection in each group infected at both initiation and at terminatio
   of study period.

Record Maintenance

All records (protocols, amendments, data sheets and final reports) are maintained in a book designated for
this study as part of the OHRI Laboratory Archives. The hard tissue specimens are also maintained in the
Archives.
Results

There was no mortality experienced during the treatment phase of this study. One sample was lost during the processing of the hard tissue samples.

Growth data are shown in Table 1221A-1. There were no significant differences observed among the groups in growth.

Smooth surface caries data are shown in Tables 1221A-2 through 1221A-4. The group treated with the placebo dentifrice developed a significantly greater number of buccal and lingual smooth surface caries, Table 1221A-2, than all of the other groups. The group treated with the SMFP/Silica dentifrice developed significantly more buccal lingual caries than the group treated with NaF/Silica dentifrice. The group treated with the Placebo dentifrice developed a significantly greater number of interproximal caries, Table 1221A-3, than the groups treated with the SNF/Silica and the NaF/Silica dentifrices. When total combined smooth surfaces caries data is considered, Table 1221A-4, the group treated with the placebo dentifrices developed a significantly greater number of total smooth surfaces carious lesions than all of the other groups. The SMFP/Silica treatment group was significantly greater in total smooth surface carious lesions than the SNF/Silica and the NaF/Silica treatment groups. There were no other statistically significant differences observed among the groups for these types of lesions.

Sulcal caries data are shown in Table 1221A-5. The group treated with the placebo dentifrice developed a significantly greater number of sulcal carious lesion than all of the other treatment groups. There were no other statistically significant differences observed among the groups for these types of lesions.

Total caries data are shown in Table 1221A-6. The group treated with the placebo dentifrice developed a significantly greater number of total carious lesion than all of the other treatment groups. The group treated with the SMFP/Silica dentifrice developed a significantly greater number of total carious lesions than the group treated with the NaF/Silica dentifrices. There were no other statistically significant differences observed among the groups for these types of lesions.

S. sobrinus data are shown in Table 1221A-7. All of the animals were infected with S. sobrinus at both initiation and termination of the study.

Conclusions

All of the U.S.P. reference standard dentifrices significantly reduced caries formation from the placebo dentifrice. In addition the NaF/Silica dentifrice was significantly greater in total caries reduction than the SMFP/Silica dentifrice.
Final Report: Study Number 1221A

Final Report Approvals

The following date and signature indicates that the Staff/Faculty Advisor has reviewed and approved the foregoing final report.

Staff/Faculty Advisor

George R. Stookey, Ph.D.
Principal Investigator

The following date and signature indicates that the Study Director has reviewed and approved the foregoing final report and the study was conducted in compliance with FDA regulations.

Study Director

Janice M. Warrick, RLATg
Director, Bioresearch Facility

The following date and signature indicates that the Quality Assurance Officer has reviewed and approved the foregoing final report. This study was inspected by the Quality Assurance Officer and reports were submitted to the Study Director as follows:

Phase
Data Audit 3/19/99
Draft Report Review 3/19/99
Report to Study Director and Management 3/19/99

This study was conducted in compliance with the Good Laboratory Practice Regulations as described in the FDA regulations part 58.

Quality Assurance

Bonny R. Bologno, B.S.
Quality Assurance Officer
## Final Report: Study Number 1221A

### Table 1221A-1: Growth

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Treatment</th>
<th>Initial Weight</th>
<th>Final Weight</th>
<th>Weight Gain</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>39</td>
<td>SMFP/Dical</td>
<td>$43.62 \pm 0.47^*$</td>
<td><strong>$143.00 \pm 2.18$</strong></td>
<td>$99.38 \pm 1.97$</td>
</tr>
<tr>
<td>E</td>
<td>40</td>
<td>Placebo</td>
<td>$43.60 \pm 0.49$</td>
<td>$141.63 \pm 2.59$</td>
<td>$98.03 \pm 2.32$</td>
</tr>
<tr>
<td>D</td>
<td>40</td>
<td>SMFP/Silica</td>
<td>$43.60 \pm 0.44$</td>
<td>$141.38 \pm 2.29$</td>
<td>$97.78 \pm 2.08$</td>
</tr>
<tr>
<td>B</td>
<td>40</td>
<td>SNF/Silica</td>
<td>$43.60 \pm 0.48$</td>
<td>$140.95 \pm 2.52$</td>
<td>$97.35 \pm 2.35$</td>
</tr>
<tr>
<td>A</td>
<td>40</td>
<td>NaF/Silica</td>
<td>$43.60 \pm 0.53$</td>
<td><strong>$140.88 \pm 2.22$</strong></td>
<td>$97.28 \pm 1.96$</td>
</tr>
</tbody>
</table>

* Standard Error of the Mean

** Values within the brackets do not differ significantly ($P > 0.05$) as determined by Duncans Multiple Range Analysis.
Table 1221A-2: Smooth Surface Caries Data  
(Buccal and Lingual)

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Treatment</th>
<th>Enamel Involvement</th>
<th>Slight Dentinal Involvement</th>
<th>Moderate Dentinal Involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>40</td>
<td>Placebo</td>
<td>$11.18 \pm 0.86^*$</td>
<td>$0.83 \pm 0.27$</td>
<td>$0.08 \pm 0.06$</td>
</tr>
<tr>
<td>D</td>
<td>40</td>
<td>SMFP/Silica</td>
<td>$6.70 \pm 0.76$</td>
<td>$0.48 \pm 0.23$</td>
<td>$0.08 \pm 0.04$</td>
</tr>
<tr>
<td>C</td>
<td>39</td>
<td>SMFP/Dical</td>
<td>$5.77 \pm 0.61$</td>
<td>$0.23 \pm 0.10$</td>
<td>$0.10 \pm 0.06$</td>
</tr>
<tr>
<td>B</td>
<td>40</td>
<td>SNF/Silica</td>
<td>$5.20 \pm 0.69$</td>
<td>$0.18 \pm 0.08$</td>
<td>$0.00 \pm 0.00$</td>
</tr>
<tr>
<td>A</td>
<td>40</td>
<td>NaF/Silica</td>
<td>$4.28 \pm 0.55$</td>
<td>$0.18 \pm 0.09$</td>
<td>$0.05 \pm 0.05$</td>
</tr>
</tbody>
</table>

* Standard Error of the Mean

** Values within the brackets do not differ significantly (P > 0.05) as determined by Duncans Multiple Range Analysis.
### Table 1221A-3: Interproximal Caries Data

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Treatment</th>
<th>Enamel Involvement</th>
<th>Slight Dentinal Involvement</th>
<th>Moderate Dentinal Involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>40</td>
<td>Placebo</td>
<td>2.75 ± 0.42**</td>
<td>0.70 ± 0.27</td>
<td>0.08 ± 0.06</td>
</tr>
<tr>
<td>D</td>
<td>40</td>
<td>SMFP/Silica</td>
<td>2.28 ± 0.40</td>
<td>0.83 ± 0.25 a</td>
<td>0.10 ± 0.08</td>
</tr>
<tr>
<td>C</td>
<td>39</td>
<td>SMFP/Dical</td>
<td>2.03 ± 0.35</td>
<td>0.36 ± 0.17</td>
<td>0.03 ± 0.03</td>
</tr>
<tr>
<td>B</td>
<td>40</td>
<td>SNF/Silica</td>
<td>1.40 ± 0.27</td>
<td>0.48 ± 0.18</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>A</td>
<td>40</td>
<td>NaF/Silica</td>
<td>1.25 ± 0.28</td>
<td>0.20 ± 0.10</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>

* Standard Error of the Mean
** Values within the brackets do not differ significantly (P > 0.05) as determined by Duncan's Multiple Range Analysis.
* Group D is significantly from group B

---

T.H. Ewing
2/25/95
### Table 1221A-4: Total Smooth Surface Caries Data
(Buccal, Lingual and Interproximal Combined)

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Treatment</th>
<th>Enamel Involvement</th>
<th>Slight Dentinal Involvement</th>
<th>Moderate Dentinal Involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>40</td>
<td>Placebo</td>
<td>13.93 ± 1.15</td>
<td>1.53 ± 0.49</td>
<td>0.15 ± 0.10</td>
</tr>
<tr>
<td>D</td>
<td>40</td>
<td>SMFP/Silica</td>
<td>8.98 ± 0.94</td>
<td>1.30 ± 0.36</td>
<td>0.18 ± 0.09</td>
</tr>
<tr>
<td>C</td>
<td>39</td>
<td>SMFP/Dical</td>
<td>7.79 ± 0.70</td>
<td>0.59 ± 0.26</td>
<td>0.13 ± 0.08</td>
</tr>
<tr>
<td>B</td>
<td>40</td>
<td>SNF/Silica</td>
<td>6.45 ± 0.77</td>
<td>0.33 ± 0.12</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>A</td>
<td>40</td>
<td>NaF/Silica</td>
<td>5.70 ± 0.66</td>
<td>0.65 ± 0.22</td>
<td>0.05 ± 0.05</td>
</tr>
</tbody>
</table>

* Standard Error of the Mean

** Values within the brackets do not differ significantly (P > 0.05) as determined by Duncans Multiple Range Analysis.
Table 1221A-5: Sulcal Caries Data

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Treatment</th>
<th>Enamel Involvement</th>
<th>Slight Dentinal Involvement</th>
<th>Moderate Dentinal Involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>40</td>
<td>Placebo</td>
<td>16.83 ± 0.96*</td>
<td>2.60 ± 0.48</td>
<td>0.38 ± 0.23</td>
</tr>
<tr>
<td>C</td>
<td>39</td>
<td>SMFP/Dical</td>
<td>14.28 ± 0.79</td>
<td>1.28 ± 0.21</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>D</td>
<td>40</td>
<td>SMFP/Silica</td>
<td>13.88 ± 0.65</td>
<td>1.90 ± 0.31</td>
<td>0.08 ± 0.04</td>
</tr>
<tr>
<td>B</td>
<td>40</td>
<td>SNF/Silica</td>
<td>12.33 ± 0.86</td>
<td>1.80 ± 0.30</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>A</td>
<td>40</td>
<td>NaF/Silica</td>
<td>12.15 ± 0.62</td>
<td>1.95 ± 0.27</td>
<td>0.05 ± 0.03</td>
</tr>
</tbody>
</table>

* Standard Error of the Mean
** Values within the brackets do not differ significantly (P > 0.05) as determined by Duncans Multiple Range Analysis.
* Group C is significantly different from group E

T.H. Ewing
2/25/99
## Final Report: Study Number 1221A

### Table 1221A-6: Total Caries Data

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Treatment</th>
<th>Enamel Involvement</th>
<th>Slight Dentinal Involvement</th>
<th>Moderate Dentinal Involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td>40</td>
<td>Placebo</td>
<td>32.75 ± 1.91*</td>
<td>4.13 ± 0.86</td>
<td>a 0.53 ± 0.28</td>
</tr>
<tr>
<td>D</td>
<td>40</td>
<td>SMFP/Silica</td>
<td>22.85 ± 1.47**</td>
<td>3.20 ± 0.57</td>
<td>0.25 ± 0.10</td>
</tr>
<tr>
<td>C</td>
<td>39</td>
<td>SMFP/Dical</td>
<td>22.08 ± 1.24</td>
<td>1.87 ± 0.38</td>
<td>0.13 ± 0.08</td>
</tr>
<tr>
<td>B</td>
<td>40</td>
<td>SNF/Silica</td>
<td>18.78 ± 1.39</td>
<td>2.13 ± 0.32</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>A</td>
<td>40</td>
<td>NaF/Silica</td>
<td>17.85 ± 1.10</td>
<td>2.60 ± 0.44</td>
<td>0.10 ± 0.06</td>
</tr>
</tbody>
</table>

* Standard Error of the Mean

** Values within the brackets do not differ significantly (P > 0.05) as determined by Duncans Multiple Rang Analysis.

a Group E is significantly different from groups B and C

T.H. Ewin
2/25/9
Table 1221A-7: *S. Sobrinus*

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Percent Animals Infected With <em>S. Sobrinus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1&lt;50*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Initiation</td>
</tr>
<tr>
<td>A</td>
<td>NaF/Silica</td>
<td>22.5</td>
</tr>
<tr>
<td>B</td>
<td>SNF/Silica</td>
<td>27.5</td>
</tr>
<tr>
<td>C</td>
<td>SMFP/Dical</td>
<td>5</td>
</tr>
<tr>
<td>D</td>
<td>SMFP/Silica</td>
<td>12.5</td>
</tr>
<tr>
<td>E</td>
<td>Placebo</td>
<td>0</td>
</tr>
</tbody>
</table>

**Termination**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Percent Animals Infected With <em>S. Sobrinus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1&lt;50*</td>
</tr>
<tr>
<td>A</td>
<td>NaF/Silica</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>SNF/Silica</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>SMFP/Dical</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>SMFP/Silica</td>
<td>0</td>
</tr>
<tr>
<td>E</td>
<td>Placebo</td>
<td>0</td>
</tr>
</tbody>
</table>

* Colony Forming Units

T.H. Ewing  
2/25/99
Appendix A. Keyes Scoring Method\textsuperscript{a,b}

The method divides the sulcal aspect of the mandibular molars into linear units: six for the first molar, four for the second, and four for the third molar. The severity scores are E, lesions present only in the enamel; D\textsubscript{S}, lesions involving the DEJ; D\textsubscript{m}, lesions extending into the dentin; and D\textsubscript{x}, which represented breakdown of the dentin. The buccal involvement is obtained by determining the number of unit area in which caries has penetrated to the E, D\textsubscript{s}, D\textsubscript{m}, D\textsubscript{x} depth.

The estimation of the sulcal scores is achieved by applying a linear estimation to theoretically flattened-out sulci and evaluating depth as indicated previously for the buccal section. The number of linear units assigned to each sulcus beginning with the first to the third molars are: 1st mandibular molar 2, 3, 2; 2nd mandibular molar 3, 2; 3rd mandibular 2; 1st maxillary molar 2, 3; 2nd maxillary molar 3; 3rd maxillary molar 2. The number of linear units assigned to each molar as well as for the buccal-lingual surface are summarized in the following table.

<table>
<thead>
<tr>
<th>Lesion Type</th>
<th>Mandibular</th>
<th>Maxillary</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st</td>
<td>2nd</td>
</tr>
<tr>
<td>Buccal</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Lingual</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Sulcal</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Proximal</td>
<td>1</td>
<td>2\textsuperscript{*}</td>
</tr>
</tbody>
</table>

\* One mesial and one distal unit

\textsuperscript{a} Navia, Juan, N.: Animal Models in Dental Research, pp 287 and 290, 1977.
### Appendix B. Composition of MIT 305

<table>
<thead>
<tr>
<th>Component</th>
<th>Percent of Composition By Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornstarch</td>
<td>62.0</td>
</tr>
<tr>
<td>Sucrose (Confectioner's Sugar)</td>
<td>5.0</td>
</tr>
<tr>
<td>Lactalbumin</td>
<td>20.0</td>
</tr>
<tr>
<td>Teklad Mineral Mix (TD 70191)</td>
<td>3.0</td>
</tr>
<tr>
<td>Vitamin Mix (Teklad 40060)</td>
<td>1.0</td>
</tr>
<tr>
<td>Cottonseed Oil</td>
<td>3.0</td>
</tr>
<tr>
<td>Cellulose (Alphacel)</td>
<td>6.0</td>
</tr>
</tbody>
</table>

| Sum                              | 100.0                            |
## Appendix C. Composition of MIT 200 Diet

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percent of Composition By Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Confectioners Sugar</td>
<td>67</td>
</tr>
<tr>
<td>Lactalbumin</td>
<td>20</td>
</tr>
<tr>
<td>Cottonseed Oil</td>
<td>3</td>
</tr>
<tr>
<td>Cellulose</td>
<td>6</td>
</tr>
<tr>
<td>Vitamin Mix (Teklad 40060)</td>
<td>1</td>
</tr>
<tr>
<td>Mineral Mix (Teklad 70191)</td>
<td>3</td>
</tr>
</tbody>
</table>

100
Appendix D. Standardized Culture

I. Preparation of Inoculum

a) **Day 1**
Nine days prior to study stratification, start a new lyophilized culture (ATCC strain #27352 *Streptococcus sobrinus*). Heat the vial over a flame and squirt a little ethanol on it to crack the glass. Wrap the vial in a paper towel (moisten paper towel with ethanol before using) and gently hit the vial with tweezers. Take out the cotton and the inner vial with sterile tweezers. Add 1 mL of BHI broth to the vial and resuspend the culture. Always flame the top of all flasks used in transferring procedures. Transfer the 1mL of suspended culture to 10mL of BHI broth in a sterile screw cap tube. Incubate overnight at 37°C in 10% CO₂.

b) **Day 2**
Using a sterile loop, check the overnight culture Gram stain and catalase activity. It should be Gram positive cocci and catalase negative. Transfer 1mL of the overnight culture to another screw cap tube containing 10mL of BHI. Incubate 37°C and 10% CO₂.

c) **Day 3**
Transfer 1mL of overnight culture to a new screw cap tube with 10mL BHI. Also transfer 1mL of overnight culture to a bottle containing 200mL BHI. Transfer another 1mL to a second 200mL bottle of BHI. Incubate 37°C and 10% CO₂.

d) **Day 4**
Transfer 1mL of the 10mL overnight culture to a new tube 10mL BHI. Incubate same as above. Transfer the 200mL bottle’s broth into two 250mL capacity centrifuge tubes. Centrifuge at 8K for 15 minutes. Pour off the supernatant and resuspend the pellets in PBS pH 7.2. Check the concentration at 375nm wavelength and adjust the O.D. to 0.7-0.8 with more PBS. Take to the Bioresearch Facility, 5th floor.

e) **Day 5**
Do the same as Wednesday. Transfer to a new 10mL BHI.

f) **Day 6**
Do the same as Thursday. Transfer 1mL each to two 200mL bottles BHI and transfer 1mL to 10mL BHI.

g) **Day 7**
Do the same as Friday with the 200mL overnight bottles. Also inoculate two more 200mL bottles with 1mL each. Inoculate another 10mL BHI screw cap tube with 1mL of the overnight culture.

h) **Day 8**
Do the same as Friday with the 200mL overnight bottles. Also inoculate two more 200mL bottles with 2mL each. Inoculate another 10mL BHI screw cap tube with 1mL of the overnight culture.

i) **Day 9**
Check the overnight culture Gram stain and catalase activity. Spin down the 200mL culture and resuspend. Check O.D. and take to the Bioresearch Facility. You do not need to prepare an overnight culture.

II. Preparation of PBS

a) Add 34.0g NaCl to 4L of DI H₂O and place on magnetic stirrer until NaCl has gone into solution.

b) Divide solution in to two flasks of 2L each.

c) Add 3.483g of K₂HPO₄ to one flask and 2.721g of KH₂PO₄ to the other flask.

d) Using a magnetic stirrer, titrate the K₂HPO₄ solution using the K₂HPO₄ solution until the pH is 7.2.

e) Autoclave the titrated solution and check pH prior to storing.

f) The autoclaved solution should be cooled to 72°F prior to inoculum preparation.
Final Report: Study Number 1221A

Table 1221A-7: S. Sobrinus

<table>
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<tr>
<th>Group</th>
<th>Treatment</th>
<th>Colony Forming Units</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>NaF / SILICA</td>
<td>22.5 15 17.5 42.5 100</td>
</tr>
<tr>
<td>B</td>
<td>SNF / SILICA</td>
<td>27.5 30 7.5 35 100</td>
</tr>
<tr>
<td>C</td>
<td>SMFP / DICAL</td>
<td>5 22.5 7.5 65 100</td>
</tr>
<tr>
<td>D</td>
<td>SMFP / SILICA</td>
<td>12.5 27.5 10 50 100</td>
</tr>
<tr>
<td>E</td>
<td>PLACEBO</td>
<td>0 0 2.5 97.5 100</td>
</tr>
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</table>

*Colony Forming Units
T.H. Ewing
3/1/99
EFU Study #121898: Comparison of the EFU performance using human and bovine enamel substrates. Enamelon, Inc., 7 Cedar Brook Drive, Cranbury, NJ 08512

Abstract

The purpose of this study is to compare the fluoride uptake performance of a USP standard fluoride toothpaste with placebo toothpaste using human and bovine enamel substrates. The test procedure was similar to the one identified as Procedure 40 in the FDA monograph. The Essential Key Elements of the protocol were summarized below:

- **Substrate:** Sound Human and Bovine enamel specimens.
- **Sample size and Controls:** each test cell contained 12 enamel samples.
- **Preconditioning:**
  - Pre-Treatment: Etch. 1 M HClO4, 15 seconds
  - Demin Solutions: 0.025M lactic + 0.0002M MHDP, 24 hours
- **Treatment Regimen:**
  - Diluent: Pooled human saliva
  - Dilution: 1:3
  - Time: 30 minutes
- **Evaluation of Substrate:**
  - Sampling: Acid etch
  - Analysis: Fluoride electrode

Enamel fluoride uptake amount were summarized in the Table below:

<table>
<thead>
<tr>
<th></th>
<th>∆ Enamel Fluoride Content (post-treatment minus pre-treatment) [a larger number indicates greater enamel fluoride uptake]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative Control</td>
</tr>
<tr>
<td>Human Enamel (Mean ± SEM, N=12)</td>
<td>28 ± 4 ppm</td>
</tr>
<tr>
<td>Bovine Enamel (Mean ± SEM, N=12)</td>
<td>21 ± 4 ppm</td>
</tr>
</tbody>
</table>

The results clearly slow the comparability of the two substrate in the enamel fluoride uptake test.
Final Report: Enamel Fluoride Uptake Study Number
FDA Method #40

Study Number

Oral Health Research Institute Number

Study Sponsor

Conducting Agency
Indiana University School of Dentistry
Oral Health Research Institute
415 Lansing Street
Indianapolis, Indiana 46202

Purpose
The purpose of this *in vitro* study was to determine the effect of dentifrices on promoting fluoride uptake into incipient enamel lesions. The test procedure was similar to the one identified as Procedure 40 in the FDA Monograph.

Procedure
Sound, upper, central, human incisors were selected and cleaned of all adhering soft tissue.

A core of enamel 3mm in diameter was prepared from each tooth by cutting perpendicular to the labial surface with a hollow-core diamond drill bit. This was performed under water to prevent overheating of the specimens. Each specimen was embedded in the end of a plexiglass rod (1/4" diameter x 2" long) using methylmethacrylate. The excess acrylic was cut away exposing the enamel surface. The enamel specimens were polished with 600 grit wet/dry paper and then with micro-fine Gamma Alumina. The resulting specimen was a 3mm disk of enamel with all but the exposed surface covered with acrylic.

Each enamel specimen was then etched by immersion into 0.5 ml of 1M HClO₄ for 15 seconds. Throughout the etch period the etch solutions were continuously agitated. A sample of each solution was then buffered with TISAB to a pH of 5.2 (0.25 ml sample, 0.5 ml TISAB and 0.25 ml 1N NaOH) and the fluoride content determined by comparison to a similarly prepared standard curve (1 ml std and 1 ml TISAB). For use in depth of etch calculation, the Ca content of the etch solution was determined by taking 50 μA and analyzing for Ca by atomic absorbion (0.05 ml qs to 5ml). These data were the indigenous fluoride level of each specimen prior to treatment.

The specimens were once again ground and polished as described above. An incipient lesion was formed in each enamel specimen by immersion into a 0.025M lactic acid/0.2mM MHDP solution for 24 hours at room temperature. These specimens were then rinsed well with distilled water and stored in a humid environment until used.
The treatments were performed using supernatants of the dentifrice slurries. The slurries consisted of 1 part dentifrice and 3 parts (w/w) distilled water. Each slurry was mixed well and then centrifuged for 10 minutes at ~10,000 rpm. The specimens were then immersed into 25 ml of their assigned supernatant with constant stirring (350 rpm) for 30 minutes. Following treatment, the specimens were rinsed with distilled water. One layer of enamel was then removed from each specimen and analyzed for fluoride and calcium as outlined above (i.e., 15 second etch). The pretreatment fluoride (indigenous) level of each specimen was then subtracted from the posttreatment value to determine the change in enamel fluoride due to the test treatment. Calculations describe in Appendix A.

Statistical Analyses
Statistical analyses were performed with a one-way analysis of variance model. The homogeneity of the variances was tested with the Bartlett-Box F at the α=0.10 level of significance. Since the assumption of homogeneous variances did not hold, a Welch test was used to determine significant differences. Since significant differences were indicated the individual means were analyzed by the Student Newman-Keuls (SNK) test.

Test Products
The test dentifrices were as follows:

<table>
<thead>
<tr>
<th>Group</th>
<th>Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>WH-11</td>
</tr>
<tr>
<td>2</td>
<td>WH-12</td>
</tr>
<tr>
<td>3</td>
<td>WH-13</td>
</tr>
</tbody>
</table>

Results
The results are shown on the attached table. All three dentifrices were significantly different from each other with WH-11 < WH-13 < WH-12.
# Final Report: Enamel Fluoride Uptake Study Number

**FDA Method #40**

## Change in Incipient Lesion Enamel Fluoride Content

<table>
<thead>
<tr>
<th>Dentine</th>
<th>Enamel Fluoride Concentration (ppm)</th>
<th>Increase</th>
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<tr>
<td></td>
<td>Pre-Treatment</td>
<td>Post-Treatment</td>
</tr>
<tr>
<td>WH-11</td>
<td>57 ± 4*</td>
<td>85 ± 4</td>
</tr>
<tr>
<td>WH-13</td>
<td>50 ± 3</td>
<td>1425 ± 56</td>
</tr>
<tr>
<td>WH-12</td>
<td>55 ± 3</td>
<td>3456 ± 144</td>
</tr>
</tbody>
</table>

* Mean ± SEM (N=12)

** Values within brackets do not differ significantly (p>0.05) as determined by Newman-Keuls analysis.

---

Gerald D. Wood  
12/18/98
Final Report: Enamel Fluoride Uptake Study Number
FDA Method #40

Change in Incipient Lesion Enamel Fluoride Content

Bovine Specimens

<table>
<thead>
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<td>58 ± 4*</td>
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<tr>
<td>WH-13</td>
<td>54 ± 2</td>
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<tr>
<td>WH-12</td>
<td>53 ± 3</td>
</tr>
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</table>

* Mean ± SEM (N=12)
** Values within brackets do not differ significantly (p>0.05) as determined by Newman-Keuls analysis.

Gerald D. Wood
12/18/98

As an additional test, the same dentifrices were run using the exact same procedures except for bovine enamel. The results are shown above and are not different from the human enamel results. In fact, the actual values are not significantly different except with dentifrice WH-13. These results indicate that bovine enamel could be used in this test with the expectation of obtaining the same results as with human enamel.
Appendix A

Fluoride calculation using the etch data

Depth = \[
\frac{\text{ppm Ca found} \times 0.5 \times 100 \times 1000}{7.07 \times 2870 \times 0.367} = \text{Ca} \times 6.7143
\]

Where

- ppm Ca = raw data
- 0.5 = converts to total Ca (use only 0.5 ml)
- 100 = dilution factor (0.05 ml q.s.to 5.0 ml)
- 1000 = convert from mm to um
- 7.07 = area of sample (3.0 mm disk)
- 2870 = density of sound enamel (ug/mm³)
- 0.367 = % Ca in sound enamel

\[
\text{F ppm} = \frac{\text{ppm F found} \times 10^6}{7.07 \times \text{depth} \times 2870 / 1000} = \frac{\text{F ppm} \times 10^6}{\text{Ca ppm} \times 136.2392}
\]

Where

- F ppm = raw data (in ug F)
- \(10^6\) = converts from ug/ug to ug/g
- 7.07 = area of sample (3.0 mm disk)
- depth = from above calculation
- 2870 = density of sound enamel (ug/mm³)
- 1000 = converts from um to mm

Reference
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<th>UPTAKE</th>
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<td></td>
<td>FDA #40</td>
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12/18/98
ENAMEL FLUORIDE UPTAKE
STUDY c. FDA #40

CHANGE IN FLUORIDE

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**MEAN** 3400.76
**STD.DEV.** 495.00
**STD.ERR.** 142.89

12/18/98
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| **MEAN**               | 21.97  | 14.53   | 4.38    |
| **STD.DEV.**           |        |         |         |
| **STD.ERR.**           |        |         |         |

12/18/98
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15 SEC

12/18/98
## Enamel Fluoride Uptake Study

**FDA #40**

### Change in Fluoride

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<tr>
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12/18/98
## ENAMEL FLUORIDE UPTAKE STUDY

### INDIVIDUAL SPECIMEN DATA

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Product 1  
**WH-11**

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12/18/98
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12/18/98
### ENAMEL FLUORIDE UPTAKE STUDY

**INDIVIDUAL SPECIMEN DATA**

**GROUP 4**

**Product 4**

**WH-11 Bovine**

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ENAMEL FLUORIDE UPTAKE STUDY

INDIVIDUAL SPECIMEN DATA

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