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THE EFFECT OF A LOW FLUORIDE DELIVERY SYSTEM ON BACTERIAL METABOLISM, BACTERIAL ATTACHMENT AND CARIES

Annual Report

September 1979

(for the period 1 September 1978 to 30 September 1979)

by

Norman Tinanoff, D.D.S., M.S.
David Camosci, B.S.

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The first goal of the low fluoride delivery system contract was to establish whether the agents to be tested were effective as a result of either inhibiting bacterial growth or killing the bacteria which were found in the oral cavity. These in vitro studies established that SnF_2 inhibited bacterial growth at 60 ppm F^- and killed bacteria at 125 ppm F^-. The other fluoride agents had these antimicrobial properties at higher concentrations. Hence, at the therapeutic release goal of 1-2 ppm, the effect of fluorides

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would not be due to killing of the organisms.

The next phase of the study was to establish the lowest level of various fluoride solutions which had an effect on the bacterial acid production of Streptococcus mutans, a known cariogenic organism which produces caries by its release of acid. The various fluorides and controls were tested at 10.0, 5.0, 1.0 and 0.5 ppm F^-. It was encouraging to find that at 1 ppm F^-, NaF, SnF_2 and Na SnF had an effect on bacterial acid production. The alteration in acid production was slight but established at even 0.5 ppm F^-. This study also established that the low levels of fluoride proportionately had a greater effect on acid production.

The electron microscopic phase of these experiments gave further encouraging results. Electron dense bodies, identified as tin by electron microprobe, found in bacterial cells exposed to 5 ppm fluoride as SnF_2, gives strong indirect evidence that fluorides, even in trace amounts, become concentrated in the bacterial cells. Furthermore, the bacterial cells exposed to 5 ppm SnF_2 showed other ultrastructural changes within the cell compatible with altered growth of these cells. These structural changes noted in the cells give us optimism that experiments presently in progress concerned with measuring bacterial growth and glucan production of bacteria exposed to low levels of various fluorides will have positive results.

The studies concerned with the release of constant fluorides from material are in progress and the initial trials have been established. Zinc oxide eugenol which had 50% SnF_2 incorporated released fluoride between 0.72 and 0.13 ppm for 19 days. More work is necessary to increase the release and continue it for a longer period.

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Summary

If a slow release agent which is incorporated into the oral cavity reduces plaque and remineralizes or prevents carious lesions from forming, it may be ideal for a soldier in combat situations. In the field, there is little access to dental care and prevention of oral disease may be given a low priority. Yet preventive dental techniques have been shown to have a dramatic impact in reducing the number of soldiers lost to a combat unit. Hence, it seems inherent that a preventive dental program, effective even without the soldier's compliance, is important for a combat effective unit.

Addition of fluorides to temporary restorations may be an effective preventive technique, and this preventive technique does not require an individual's cooperation. Fluorides in the form of water fluoridation and topical treatments have had dramatic success in reducing incidence of decay, even though the mechanisms by which they work is still unclear. Studies by this author have shown that one of the actions of fluoride may be the alteration in sorption of bacteria to tooth enamel, thus preventing plaque accumulation on teeth. From the previous U.S. Army contract studies on fluoride mouthrinse, it was established that SnF_2 dramatically reduced the number of bacteria attached to enamel in vitro (see appendix E); and even in clinical trials, SnF_2 was found to reduce plaque on teeth by 50% (see appendix C, "Clinical trial to test antiplaque effect of SnF_2 mouthrinse").

From these antiplaque findings with SnF_2 when used as a mouthrinse, it seemed necessary to test if a fluoride compound could be placed permanently in the oral cavity with the strategy of being slowly released so that it would

have a constant beneficial effect on oral health and require no compliance or thought by the individual it was used on. Studies such as the present one were conducted to explore the mechanisms, regimen and benefits of such a possible preventive therapeutic procedure.

The first goal of the low fluoride delivery system contract was to establish whether the agents to be tested were effective as a result of either inhibiting bacterial growth or killing the bacteria which were found in the oral cavity. These studies established that SnF_2 inhibited bacterial growth at 60 ppm F^- and killed bacteria at 125 ppm F^-. The other fluoride agents had these antimicrobial properties at higher concentrations. Hence, at the therapeutic release goal of 1-2 ppm, the effect of fluorides would not be due to an inhibition or killing of the organisms.

The next phase of the study was to establish the lowest level of various fluoride solutions which had an effect on the bacterial acid production of Streptococcus mutans, a known cariogenic organism which produces caries by its release of acid. The various fluorides and controls were tested at 10.0, 5.0, 1.0 and 0.5 ppm F^-. It was encouraging to find that at 1 ppm F^-, NaF, SnF_2 and Na_2SnF_6 had an effect on bacterial acid production. The alteration in acid production was slight but established at even 0.5 ppm F^-. This study also established that the low levels of fluoride proportionately had a greater effect on acid production.

The electron microscopic phase of these experiments gave further encouraging results. Electron dense bodies, identified as tin by electron microprobe, found in bacterial cells exposed to 5 ppm fluoride as SnF_2, gives strong indirect evidence that fluorides, even in trace amounts, become concentrated in the bacterial cells. Furthermore, the bacterial cells exposed to 5 ppm SnF_2 showed other ultrastructural changes within the cell compatible with altered

growth of these cells. These structural changes noted in the cells give us optimism that experiments presently in progress concerned with measuring bacterial growth and glucan production of bacteria exposed to low levels of various fluorides will have positive results.

The atomic absorption spectrophotometic studies in progress will quantitate the amount of tin within cells exposed to SnF_2 and then establish how much tin is found in the bacterial cell and how much tin is found in the extracellular material. These studies, which are in progress, were not listed in the original contract, but as the other studies progressed, they seemed essential.

The studies concerned with the release of constant fluorides from material are in progress and the initial trials have been established. Zinc oxide eugenol which had 50% SnF_2 incorporated released fluoride between 0.72 and 0.13 ppm for 19 days. More work is necessary to increase the release and continue it for a longer period.

The plan of work for year 2 of the Low Fluoride Delivery System Contract includes completing the atomic absorption experiments, completing the glucan and DNA experiments and optimizing the slow release of fluoride from the temporary material. Also to be initiated and completed during this time will be the hamster animal trials in which plaque indices, caries, total number of organisms on teeth, plus S. mutans recovery in animals which have slow releasing fluorides present.

M.I.C. AND M.L.C. OF FLUORIDE SOLUTIONS

Introduction

For quantitation of the antimicrobial effects of the various test agents, dilutions of fluorides or controls were incorporated at various dilutions into broth containing a standardized suspension of Streptococcus mutans. After appropriate incubation, the minimal inhibiting concentration (MIC) was determined as the lowest concentration that would inhibit growth of the microorganisms. The minimal lethal concentrations (MLC) was determined by transferring an aliquot from the tubes showing no growth to agar plates which were incubated to see if any organisms were viable.

The purpose of this experiment was to rule out the possibility that various fluorides may have antiplaque properties at low concentrations due to their ability to kill or suspend growth of plaque forming bacteria.

Materials and Methods

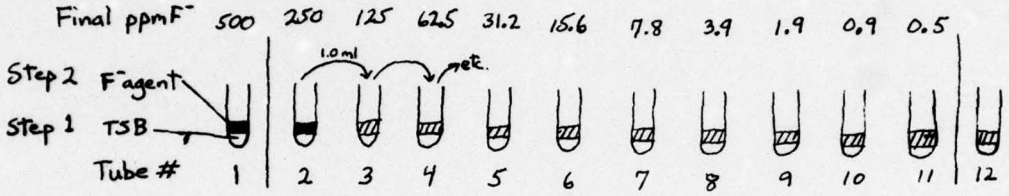
Streptococcus mutans NCTC 10449 was adapted to growth in trypticase soy broth (TSB; BBL) and then 0.1 ml of the adapted strain was transferred to 10 ml of the medium. The turbidity of the growth was standardized and diluted 1:200 to produce an inoculum containing approximately 5.0×10^5 CFU/ml.

The potential antimicrobial agents, SnF_2, $SnCl_2$, TiF_4, Na_2SnF_6 and NaF, were diluted with 1 ml of bacteria medium such that each test tube contained 1/2 the concentration of the agent from the previous tube (Figure 1). To these tubes containing serial dilutions, 1 ml of the standardized cell suspension was added. The tubes containing the various dilutions of antimicrobial agent plus the inoculum were vortexed, and incubated at 35° for 16-18 hours and read.

A large cluster of growth or definite turbidity was considered evidence that the agent failed to inhibit growth at that concentration due to precipi-

tates of some of the test agents, the appearance of the positive and negative control tubes was considered before determining the end point. The MLC was determined by transferring 1.0 ml of broth from each tube showing inhibited growth to blood agar plates and to mitis salivarius plates. The plates were inspected for growth of S. mutans after 2 days incubation at 37^{0}.

Fig. 1 Procedure for MIC and MLC Test Run



Step 1 Add 1.0 ml TSB to all tubes.

Step 2 Add agent ie.1000 ppm F- to # 1 and 2 tubes and 2-fold dilute starting at #2 and ending at #11.

Step 3 Add 1.0 ml inoculum of standarized suspension to all tubes except #1.

Results

As shown in Tables 1-5 representing the various fluoride agents tested for their MIC and MLC effect upon S. mutans, SnF_2 produced the lowest ppm fluoride in concentration needed for both the minimal inhibitory and minimal lethal effect. Table 6 summarizes the MIC and MLC results obtained from all the agents.

RESULTS

SnF_2 Results

ppm F^-	Culture Vial	MSS plate	BA plate
250	NG, P	NG	NG
125	NG, P	NG	NG
100	NG, P	<<G	<<G
90	NG, P	<G	<G
80	NG, P	<G	<G
70	NG, P	<G	<G
60	NG, P	G	G
50	G, P	G	G
40	G, P	G	G
30	G, P	G	G
20	G, P	G	G
10	G, P	G	G
5	G, P	G	G
2.5	G, NP	G	G
1.8	G, NP	G	G
0.9	G, NP	G	G
0.5	G, NP	G	G

Key: NG = No Growth
<G = Less Growth
G = Growth
P = Precipitate
NP = No Precipitate

Table 1: Dilutions of SnF_2 ranging from 0.5 to 250 ppm F^- were evaluated for a minimum inhibitory concentration (MIC) and a minimum lethal concentration (MLC). The MIC of SnF_2, determined by the culture vial of the lowest dilution showing no growth, was 60 ppm F^-. The MLC of SnF_2, determined by the lowest concentration on BA and MSS plates showing no growth, was 125 ppm F^-. Plated dilutions of SnF_2 ranging from 60 ppm F^- or less showed no difference in the amount of growth after 24 hours; while plated dilutions of 70 ppm F^- or greater showed a decrease in growth. None of the control vials or plates exhibited growth. No contamination was noted on culture plates of test dilutions. Also, a milky white precipitate was present in both the control and test dilutions at 5 ppm F^- or above.

$SnCl_2$ Results

ppm Sn*	Culture Vial	MSS plate	BA plate
1000	NG, P	NG	NG
900	NG, P	NG	NG
800	NG, P	NG	NG
700	NG, P	NG	NG
600	NG, P	NG	NG
500	NG, P	NG	NG
400	NG, P	NG	NG
300	NG, P	NG	NG
250	NG, P	NG	NG
225	NG, P	NG	NG
200	NG, P	G	G
175	<G, P	G	G
150	G, P	G	G
125	G, P	G	G
100	G, P	G	G
75	G, P	G	G
50	G, P	G	G

Key: NG = No Growth
<G = Less Growth
G = Growth
P = Precipitate

*ppm equivalent to Sn^{++} in a SnF_2 solution of equal concentration

Table 2: Dilutions of $SnCl_2$ ranging from 50 to 1000 ppm Sn^{++}, equivalent to the Sn^{++} in a SnF_2 solution of equal concentration, were evaluated for a minimum inhibitory concentration (MIC) and a minimum lethal concentration (MLC). The MIC of $SnCl_2$, determined by the culture vial of the lowest dilution showing no growth, was 200 ppm F^-. The MLC of $SnCl_2$, determined by the lowest concentration on BA and MSS plates showing no growth, was 225 ppm F^-. None of the control vials or plates exhibited growth. No contamination was noted on culture plates of test dilutions. Also, a cloudy yellow-white precipitate was present in all control and test dilution vials.

TiF_4 Results

ppm F^-	Culture Vial	MSS plate	BA plate
1000	NG, P	NG	NG
900	NG, P	NG	NG
800	NG, P	NG	NG
700	NG, P	NG	NG
650	NG, P	NG	NG
625	NG, P	NG	NG
600			
575±25	NG, P	NG	NG
550±25	NG, P	G	G
525			
500	G, P	G	G
475	G, P	G	G
450	G, P	G	G
400	G, P	G	G
300	G, P	G	G
200	G, P	G	G
100	G, P	G	G

Key: NG = No Growth
G = Growth
P = Precipitate

Table 3: Dilutions of TiF_4 ranging from 100 to 1000 ppm F^- were evaluated for a minimum inhibitory concentration (MIC) and a minimum lethal concentration (MLC). In all test runs, the MIC and MLC was found within the 525 to 600 ppm F^- range. The MIC of TiF_4, determined by the culture vial of the lowest dilution showing no growth, was 550±25 ppm F^-. The MLC of TiF_4, determined by the lowest concentration on BA and MSS plates showing no growth, was 575±25 ppm F^-. None of the control vials or plates exhibited growth. No contamination was noted on culture plates of test dilutions. Also, a cloudy gray-white precipitate was present in all test and control dilutions.

Na_2SnF_6 Results

ppm F^-	Culture Vial	MSS plate	BA plate
1000	NG	NG	NG
900	NG	NG	NG
800	NG	NG	NG
700	NG	NG	NG
675	NG	NG	NG
650	NG	<G	<G
625	NG	<G	<G
600	NG	G	G
575	<G	G	G
550	G	G	G
525	G	G	G
500	G	G	G
475	G	G	G
400	G	G	G
300	G	G	G
200	G	G	G
100	G	G	G

Key: NG = No Growth
<G = Less Growth
G = Growth

Table 4: Dilutions of Na_2SnF_6 ranging from 100 to 1000 ppm F^- were evaluated for a minimum inhibitory concentration (MIC) and a minimum lethal concentration (MLC). The MIC of Na_2SnF_6, determined by the culture vials of the lowest dilutions showing no growth, was 600 ppm F^-. The MLC of Na_2SnF_6, determined by the lowest concentration on BA and MSS plates showing no growth, was 675 ppm F^-. None of the control vials or plates exhibited growth. No contamination was noted on culture plates of test dilutions.

NaF Results

ppm F^-	Culture Vial	MSS plate	BA plate
5000	NG	NG	NG
4500	NG	NG	NG
4000	NG	NG	NG
3500	NG	NG	NG
3250	NG	NG	NG
3000	NG	NG	NG
2750	NG	<<<G	<<<G
2500	NG	<<<G	<<<G
2000	NG	<<G	<<G
1500	NG	<<G	<<G
1000	NG	<G	<G
900	NG	<G	<G
800	NG	<G	<G
700	NG	<G	<G
600	NG	<G	<G
500	NG	<G	<G
400	NG	G	G
350	NG	G	G
300	NG	G	G
250	<G	G	G
200	G	G	G
150	G	G	G
100	G	G	G
50	G	G	G

Key: NG = No Growth
<G = Less Growth
G = Growth

Table 5: Dilutions of NaF ranging from 50 to 5000 ppm F^- were evaluated for a minimum inhibitory concentration (MIC) and a minimum lethal concentration (MLC). The MIC of NaF, determined by the culture vial of the lowest dilution showing no growth, was 300 ppm F^-. The MLC of NaF, determined by the lowest concentration on BA and MSS plates showing no growth, was 3000 ppm F^-. Plated dilutions of NaF ranging from 500 to 2750 ppm F^- exhibited decreased growth on BA and MSS plates after 24 hours incubation at 35° C. None of the control vials or plates exhibited growth. No contamination was noted on culture plates of test dilutions.

TABLE 6

Summary of MIC and MLC Test Results

Test Compound	MIC (ppm F^-)	MLC (ppm F^-)
SnF_2	60	125
$SnCl_2$*	200	225
TiF_4	550±25	575±25
Na_2SnF_6	600	675
NaF	300	3000

*ppm equivalent to Sn^{++} in a SnF_2 solution of equal concentration

Alteration in Bacterial Acid Production

Due to Various Fluoride Compounds

Introduction

The fact that dental caries is the result of acids produced by bacteria adherent to teeth is now well established. As this metabolic by-product accumulates in the bacterial plaque, a critical pH (believed to be 5.5, though no experimental evidence is shown) is reached in which enamel dissolution ensues.

Recently Miller (Miller, L., Grad. Thesis, Univ. of Iowa, ND, 547, 1974) examined how various concentrations of fluoride alter both acid production and glucose uptake in Streptococcus faecalis. Results show a decrease in bacterial acid production even at the 0.5 ppm F^- range. However, there was no inhibition of glucose transport across the cell membrane until at least 10 ppm F^- was used. Therefore, the range in our present acid production experiments is from 0.5 ppm F^- to 10.0 ppm F^- for all of the fluoride compounds to be tested.

Methods and Materials

A stock culture of Streptococcus mutans NCTC 10449 (serotype c), known to be a good plaque former in vitro (Infect. Immun. 10:189-196, 1974) and known to be carriogenic (Tanzer, J.M., personal communication) was maintained by monthly transfers in thioglycollate medium Brewer Modified (BBL) supplemented with excess $CaCO_3$. For each experiment, the culture was adapted to growth in the complex medium of Jordon et al. (J. Dent. Res. 39:116-123, 1960), supplemented with 50 mg. of Na_2CO_3 per liter and containing 5% sucrose.

The total procedure for preparing the culture used in the experiments is given in Figure 2. This insures an adapted log-phase innoculum added to the experimental culture flasks.

The five fluoride compounds tested for their ability to cause an adverse effect on bacterial acid production were NaF, SnF_2, Na_2SnF_6, Na_2PO_3F and TiF_4. The controls used in the experiments were deionized water for the fluoride control and $SnCl_2$ for the potential effect of the tin ion alone when compared to SnF_2. All the test compounds were prepared at 250 ppm F^- in 200 ml of deionized HoH and an aliquot of either 8.0, 4.0, 0.8, or 0.4 ml were added to the culture flasks to dilute the concentration of fluoride to 10, 5.0, 1.0 or 0.5 ppm F^- respectively.

The culture flasks were placed in a shaker water bath at 37° and shaken at a rate of 60 cpm. pH measurements were taken every 12 hrs. during the 48 hr. growth span. The experiment was terminated at 48 hrs. because of the constant stability of pH measurements recorded after this time.

Fluoride electrode measurements were performed for quality control of theoretical fluoride levels in the growth medium and cultures of S. mutans were plated on BAP and MS plates for purity checks.

To show whether the F^- compounds exerted a buffering effect at the acidic pH range, all compounds were added in 10 ppm F^- concentrations into a solution with a pH of 4.0 and pH changes were measured one hour later.

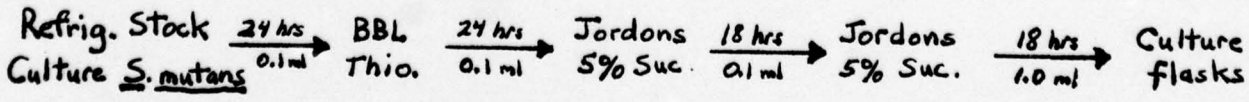


Figure 2: Procedure for adapting experimental cultures of S. mutans to a 5% sucrose environment.

Results

With regard to the ability of the five fluoride compounds to cause an adverse effect on bacterial acid production, NaF, SnF_2, and Na_2SnF_6 appear to be equally effective in producing the slowest fall in pH measurements and acquiring the highest terminal pH (Figs. 3, 4, 5, 6). There was a consistent pattern among these compounds at a F^- ion concentration of 0.5, 1.0, 5.0 and 10.0; however, this effect was not linear in proportion to the increase of F^- ion compared to the effect on decreasing the bacterial acid production (Fig. 7).

The other test agents were TiF_4 which produced an intermediate effect on decreasing acid production, and Na_2PO_4F which created a slight effect on pH measurements when added to the medium in quantities greater than 5.0 ppm F^- (Fig. 4, 5). The acid production and the terminal pH measurements of $SnCl_2$ supplemented media were closely associated to the measurements of the water control and thus considered ineffective in altering acid production.

All experiments were performed in duplicate runs and the data consolidated into one figure.

Initial fluoride electrode measurements of the culture medium containing either 5.0, 1.0 or 0.5 ppm F^- of the experimental compounds revealed the amounts of fluoride levels expected according to the theoretical formulations.

To show whether any fluoride compounds did exert a buffering effect at the acidic pH range, concentrated HCl was diluted in water to a pH of 4.0, and 10 ppm F^- for each compound was added separately to the acidic solutions. One hour later, pH measurements of the five fluoride solutions showed a slight acidic shift of a few tenths from the initial pH reading of 4.0 with the exception of Na_2PO_3F which showed a basic shift to pH 5.44.

All culture flasks were free of contamination at all times as shown by streaking blood agar plates at the end of all experimental runs.

Discussion

Recently Miller (I.A.D.R. Abstract #1010, Miami, Florida, 1976) and Kashket al al. (Caries Res. 11:301-307, 1977) examined how various concentrations of fluoride (NaF) alter both acid production and glucose uptake in S. faecalis and S. mutans. Results show a decrease in bacterial acid production even at the 0.5 ppm F^- range. However, there was no inhibition of glucose transport across the cell membrane until at least 10 ppm F^- was used. Hamilton (Caries Res. 11 (Suppl. 1):262-291, 1977) used a chemostat to show the effect of F^- ions on the glycolytic pathway of S. mutans when grown in glucose medium. The enzyme, enolase, is prevented from catabolizing the reaction of 2^- phosphoglyceric acid to PEP. PEP is needed to drive many other reactions required for cell metabolism, thereby causing other adverse effects; one example being active transport through the cell membrane. This reaction has also been shown to occur when S. mutans was grown in sucrose medium as its sole carbohydrate source (Slee, A., personal communication). In light of these experiments, the range in our present bacterial acid production experiments is from 0.5 to 10.0 ppm F^- for all of the fluoride compounds to be tested.

The alteration of lactic acid production by S. mutans seems to be due to the free fluoride ion released from the compound rather than the effect of the combined elements of the compound. All of the fluoride test compounds had some adverse effects on acid production in varying degrees which may be attributed to the ability of the compound to cross the cell membrane of the microorganism. Compounds whose molecular configurations necessitate a need for greater active transport energy derived from the cell to facilitate

cell membrane passage will be entering the cell at a lower net rate than compared to compounds which require less active transport energy. However, since fluoride alone has been shown to passively enter into the cell, the element which binds with the fluoride may be an important factor concerning entry through the cell membrane. This can be demonstrated by the compound Na_2PO_3F which cannot be accurately measured by the fluoride electrode because of the strong covalent bonds within this compound. For this reason, free fluoride ions are in low quantities which may explain the only slight adverse effect caused by this compound upon bacteria acid production. TiF_4 being hydroscopic, makes this compound too unstable for practical applicational purposes and the compound tends to lose 1 or 2 ppm F^- ions when prepared from the theoretical formula as shown by fluoride electrode measurements during quality control checks.

The fluoride compounds themselves were shown to produce minor pH changes when tested in the acidic range of pH 4.0. Only Na_2PO_3F showed a strong basic shift of 1.5 pH units from the control. However, this compound had little or no effect on the terminal pH produced by the microorganisms when compared to the non-fluoridated water control. Hence the addition of the test compounds to the media had no pH effect in the trials with the microorganisms and the pH changes produced by the fluoride per se can be ignored.

The purpose of these experiments was to determine the lowest concentration of various fluoride compounds which would alter acid production by a known cariogenic microorganism. The experiments have shown that the fluoride ion, even at 0.5 ppm has a measurable effect on bacterial acid production and at 10 ppm F^-, this reduction of acid production is approximately 0.6 pH units. Is this reduction of acid production, i.e., 0.6

moles acid/liter, clinically significant?

Opinions within the field of physical chemistry state the critical pH value to enamel demineralization to be somewhere in the range of 4.5-5.5. Since the range falls within the terminal pH measurements obtained by our present investigations of F^- compounds exposed to S. mutans, this information is important in determining the quantities of fluoride needed to be released by the temporary restorations and whether the effects noted in these experiments will stop acid demineralization of teeth. Our laboratory is presently measuring by atomic absorption spectrophotometry the amount of Ca^{++} released from enamel at various pH's to further determine the critical significance of the reduced acid production found in these experiments.

NaF, SnF_2 and Na_2SnF_6 repeatedly showed in all ppm ranges the greatest effect by probably interfering with the microorganisms' glycolytic cycle and thereby reducing the ability of the cells to produce lactic acid. The greatest effect is achieved after 24 hrs. and tends to stabilize thereafter. At 48 hrs., the differences between the test agents to stabilize is probably the result of poor growth conditions of the organisms which affect the amount of acid production more than the fluoride ions at this time. However, it is interesting to note that even though there is essentially no growth of organisms at 48 hours, the terminal pH of the cultures containing fluoride is higher than those not containing fluoride.

The results of this study has looked at the effect of fluoride compounds on bacterial acid production. It may have importance if these compounds are able to maintain the pH value produced by dental plaque above the critical pH needed for enamel dissolution. However, the great importance of these fluoride compounds may lie in their ability to produce anti-

adhesion properties causing the reduction of dental plaque organisms and thereby also reducing the amount of organisms able to produce acid.

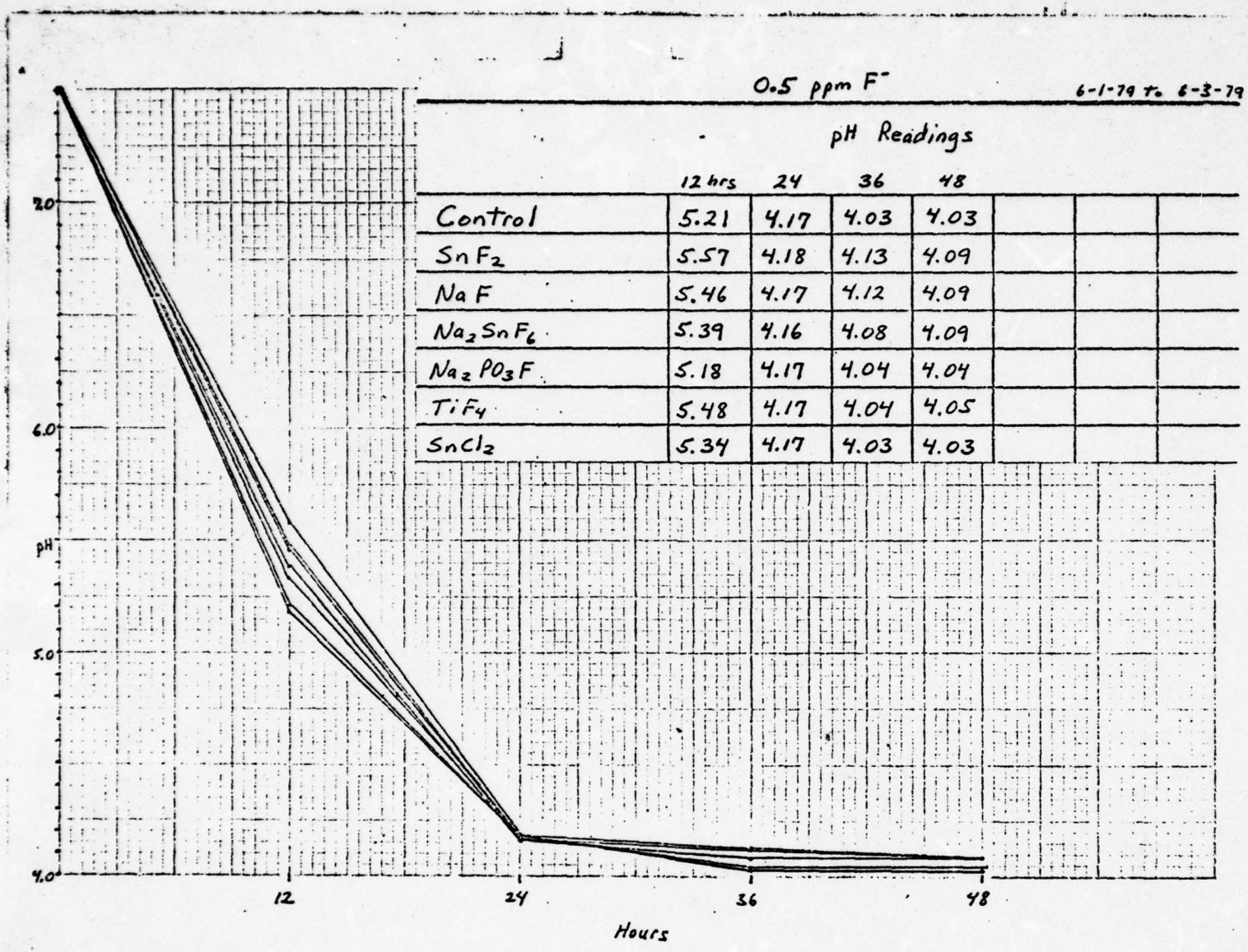


Figure 3: The effect of 0.5 ppm F^- on the acid production of S. mutans NCTC 10449 in Jordan's medium supplemented with 5% sucrose. $SnCl_2$ was included as a control for the tin effect.

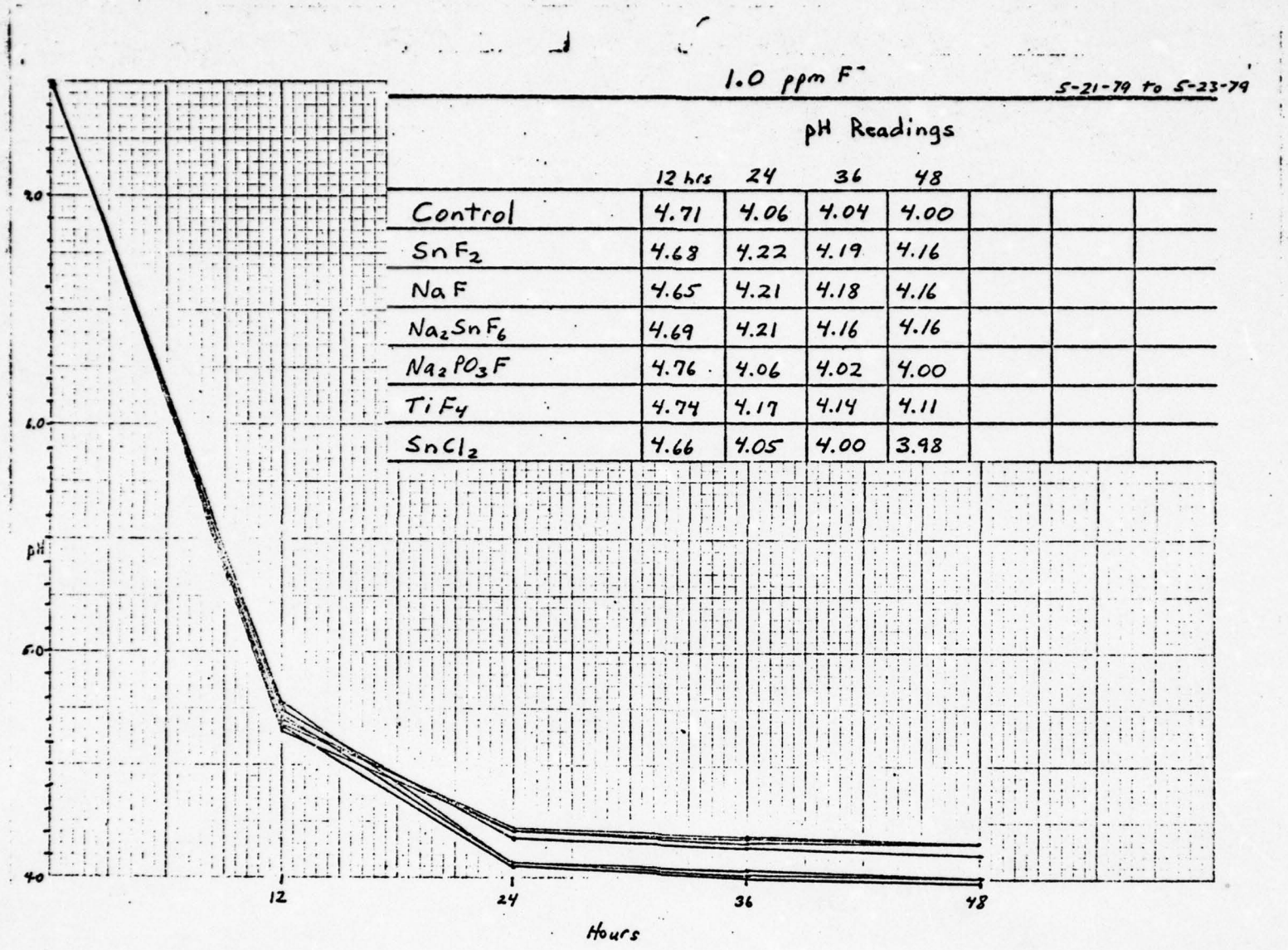


Figure 4: The effect of 1.0 ppm F^- on the acid production of S. mutans NCTC 10449 in Jordan's medium supplemented with 5% sucrose. $SnCl_2$ was included as a control for the tin effect.

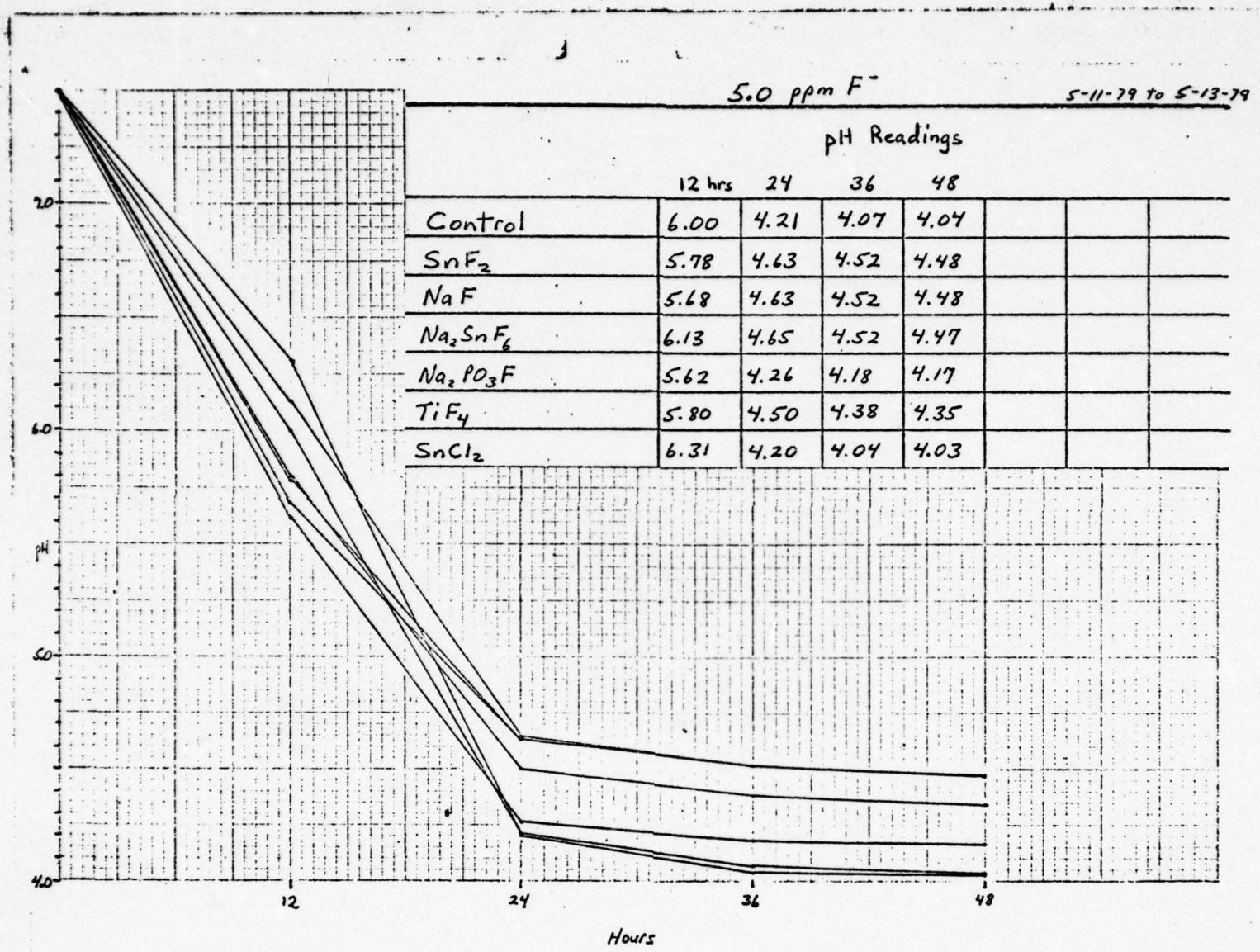


Figure 5: The effect of 5.0 ppm F^- on the acid production of S. mutans NCTC 10449 in Jordan's medium supplemented with 5% sucrose. $SnCl_2$ was included as a control for the tin effect.

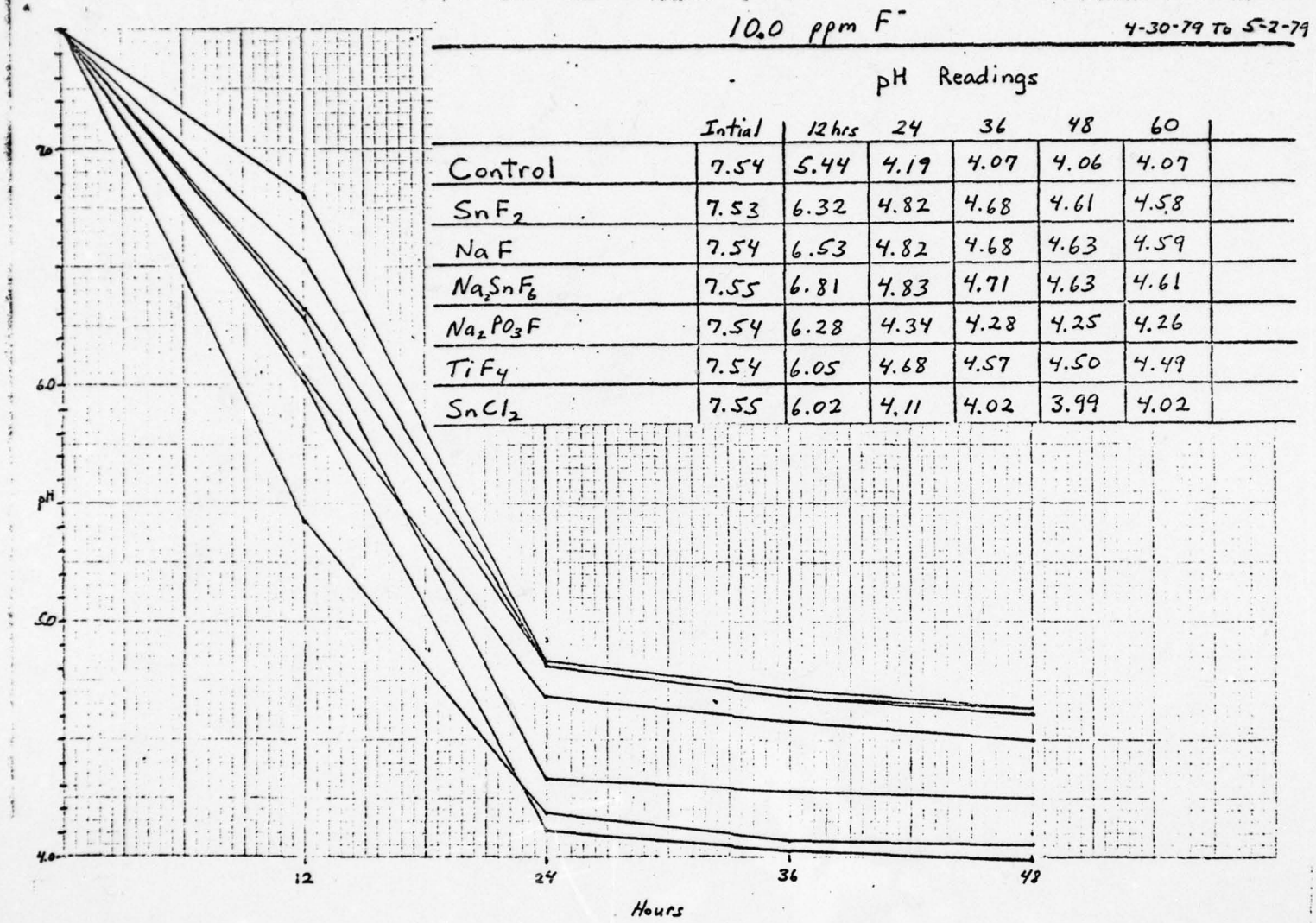


Figure 6: The effect of 10.0 ppm F^- on the acid production of S. mutans NCTC 10449 in Jordan's medium supplemented with 5% sucrose. $SnCl_2$ was included as a control for the tin effect.

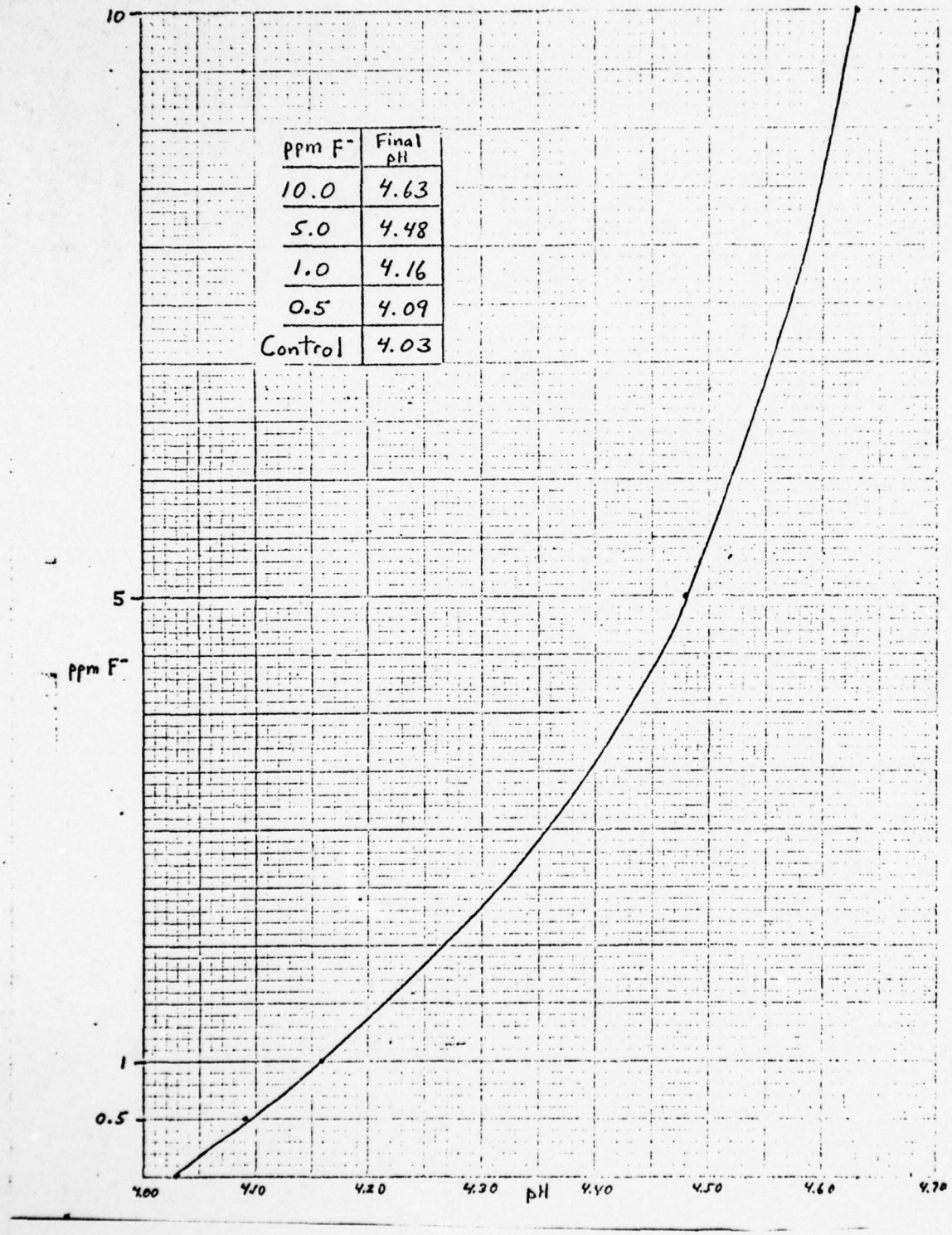


Figure 7: Summary of trials showing the effect of various fluoride concentrations on terminal pH produced by S. mutans NCTC 10449. The fluoride compounds of NaF, SnF_2 and Na_2SnF_6 produced the same terminal pH measurements. Bacterial growth was performed in Jordan's medium supplemented with 5% sucrose and incubated at 37° for 48 hours.

Electron Microscopic Evaluation of a

Low Fluoride Delivery System

Introduction

The electron microscopic phase of these experiments was utilized to try to observe the mechanisms which low levels of various fluorides alter bacterial attachment and/or metabolism. Previous experiments to observe the effects of different fluorides on plaque has successfully shown mechanisms by which fluoride alters plaque formation. In vivo experiments (Caries Res. 10:415-426, 1976) have shown that intermittent exposures of NaF appeared to have little influence on 2-day plaque formation. In these experiments, however, SnF_2 showed drastic reductions in plaque which electron microscopically appeared to be due to alteration of adhesion of bacteria to enamel and bacteria to bacteria.

The preceding U.S. Army Contract utilized transmission electron microscopy to further observe the effect of intermittent exposure of fluorides to plaque bacteria in vitro. Here electron microscopy of the bacteria-enamel interface of S. mutans subjected to 250 ppm fluoride as SnF_2 showed morphological alterations of the bacteria and extracellular material. Bacteria in these specimens showed separation from the enamel surface. The extracellular material appeared to be dissociated from the bacteria and the bacteria demonstrated morphological alterations with indistinct cell walls and the presence of intracellular vacuoles compatible with unbalanced bacterial growth (Fig. 8).

Of most interest was the presence of electron-dense bodies in or on the bacterial cells even in unstained sections (Fig. 9). Electron probe elemental analysis (Fig. 10,12) has confirmed that these electron dense bodies are essentially tin. Since bacterial specimens subjected to $SnCl_2$ treatment did not show electron dense bodies in the cells, these results indicate that both tin and fluoride are essential for the antiplaque effects found *in vivo* and *in vitro* with SnF_2. This finding may be explained by the fact that fluoride is known to become concentrated in plaque bacteria cells. Apparently as fluoride is absorbed into the cells, the associated tin ions of SnF_2 are "carried" along and hence accumulate along with the fluoride.

Figure 8 : S. mutans NCTC 10449 attached to human tooth enamel during 1 day incubation in Jordan's medium supplemented with 5% sucrose. Twice a day the enamel specimen was removed from the growth medium and suspended in SnF_2 solution (250 ppm F^-) for 1 minute, washed, and then replaced in growth medium. Note: bacteria separated from the enamel (E); extracellular polymers appear abnormally fibrillar, as well as lack of association with the bacteria (black arrows); and holes compatible with polyphosphates indicate unbalanced bacteria growth. X 10,000

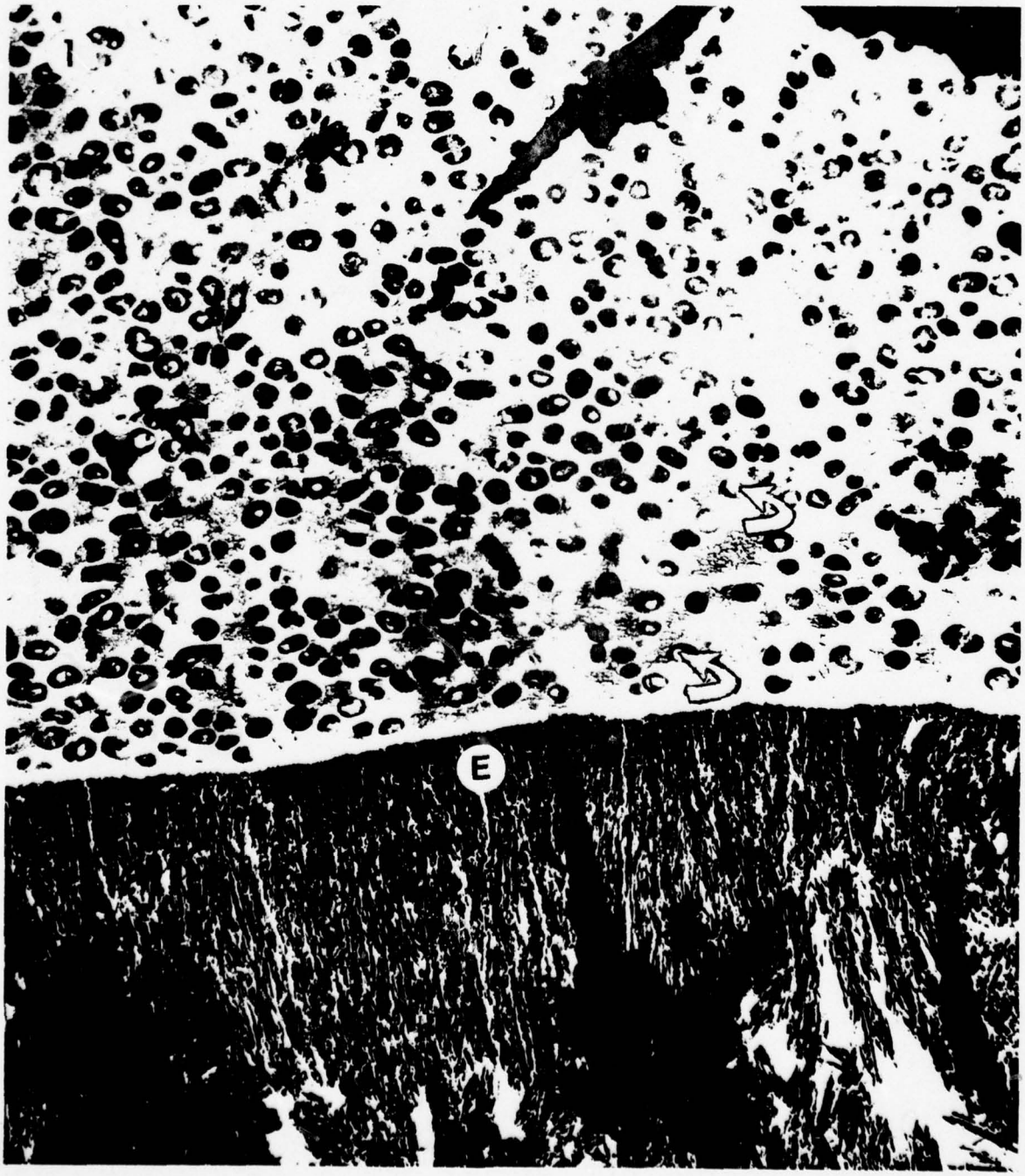


Figure 9: Higher magnification electron micrograph of Figure 8. (Structural details absent because specimen unstained). Electron dense structures can be noted within the bacterial cells. Transmission electron microprobe elemental analysis was performed on 3 selected areas (A, B, C) to enable determination of chemical composition. X 60,000

Figure 10: Electron probe of area "A" in Figure 9. This area is high in the elements tin and calcium (arrow).

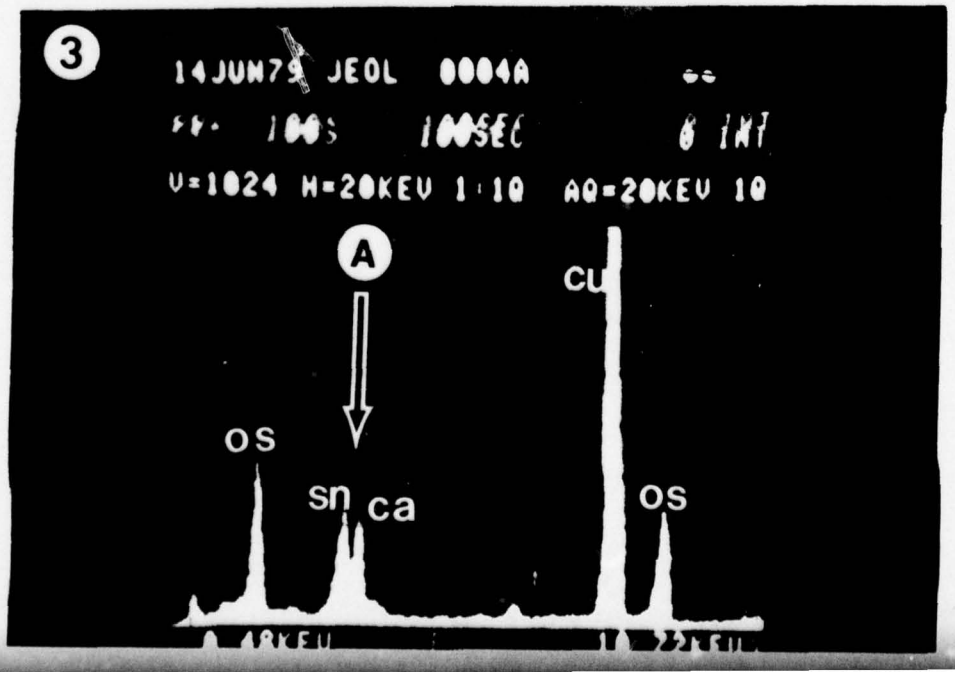


Figure 11: Electron probe of area "B" in Figure 9. This area is high only in the element calcium (arrow).

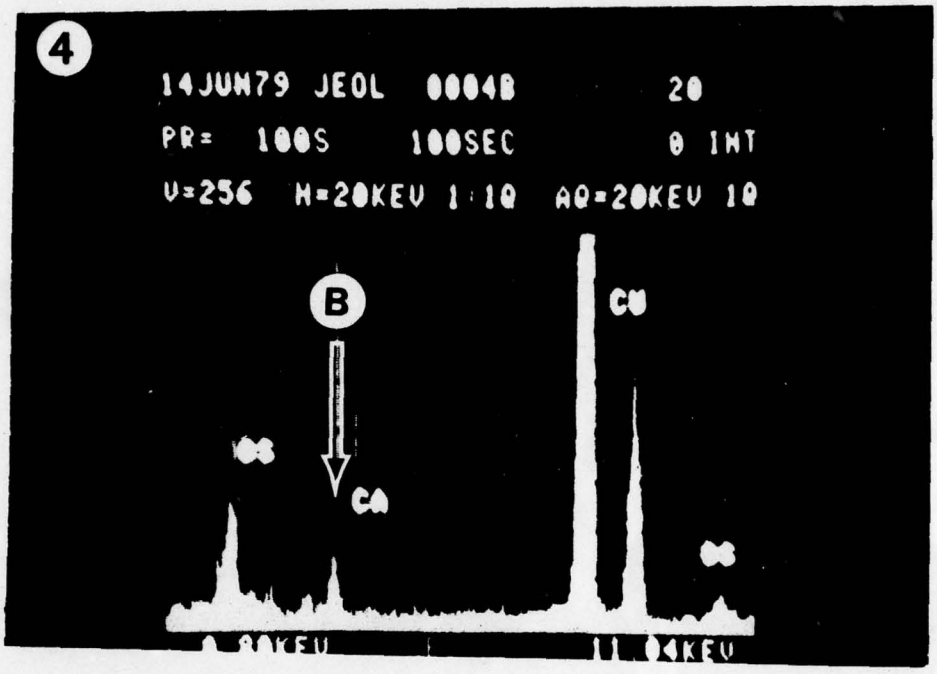


Figure 12: Electron probe of area "C" in Figure 9. This area is absent of both calcium and tin (arrow).

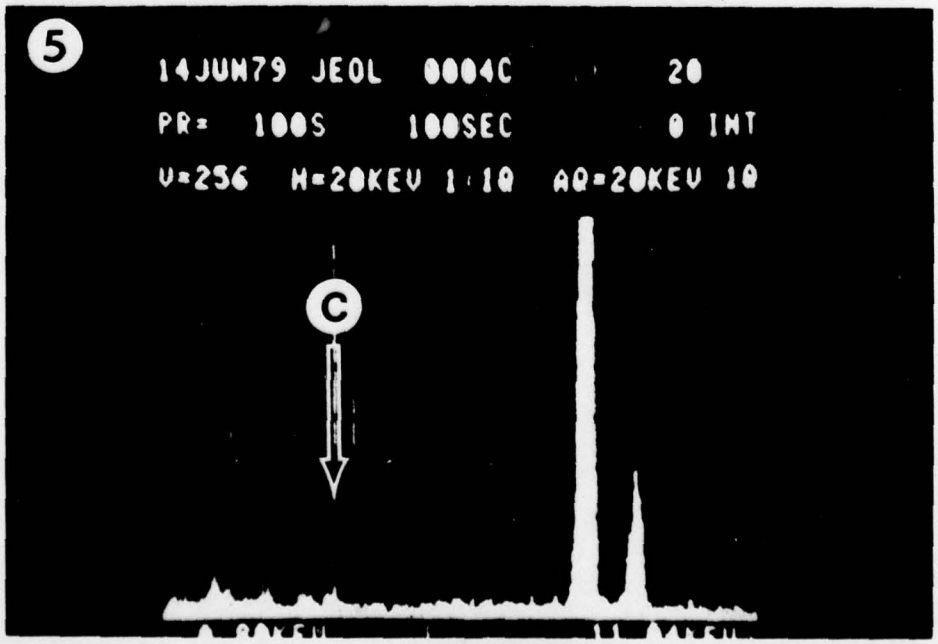


Figure 13: S. mutans 10449 incubated for 1 day in Jordan's medium with 5% sucrose. Note normal appearance of cells as shown by intact cell walls, cell membranes, and frequent cell divisions. Extracellular material is abundant and associated with the cells (arrow). X 60,000

Figure 14: S. mutans 10449 incubated for 1 day as above, yet with the addition of NaF (5 ppm F^-). Cells and extracellular material appear normal. X 60,000

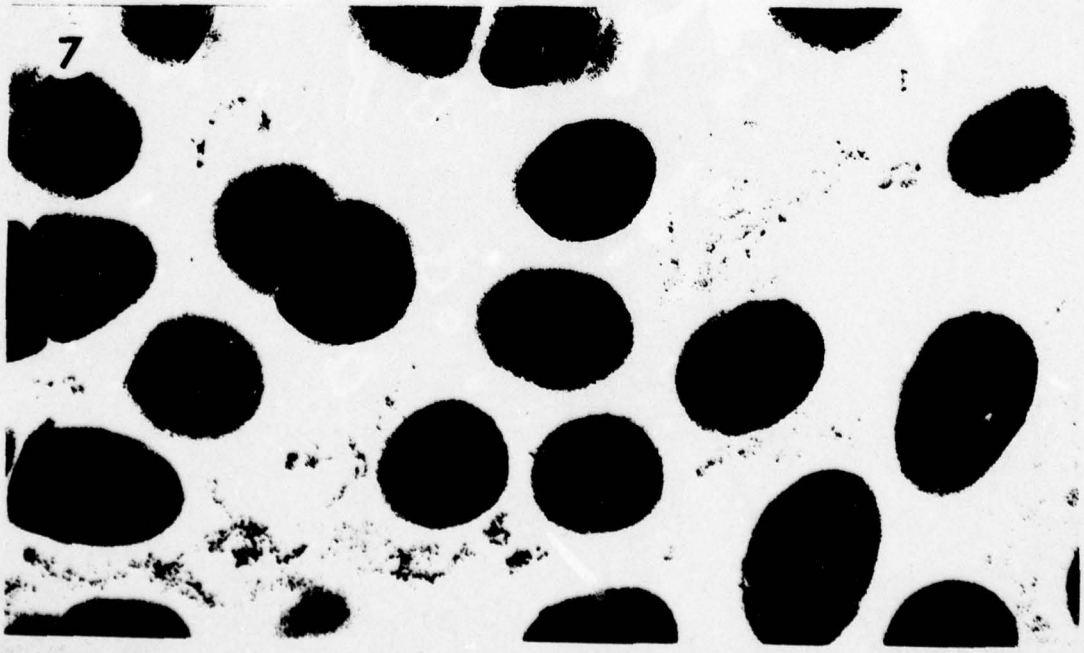
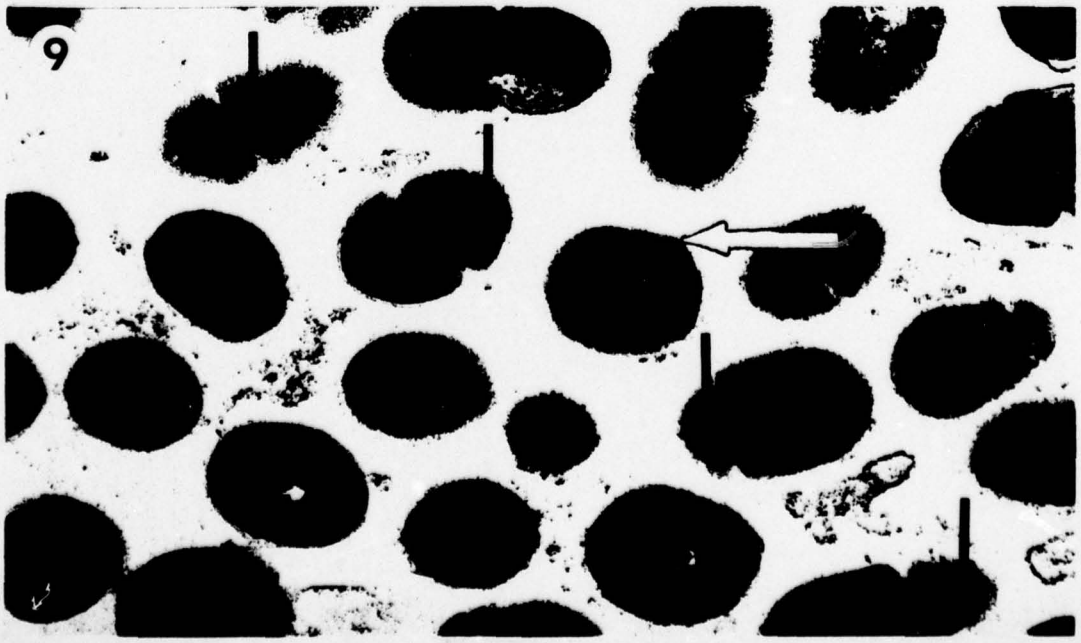


Figure 15: S. mutans 10449 incubated for 1 day with the addition of $SNCl_2$ (Sn^{++} = Sn^{++} in SnF_2, 5 ppm F^-). Cells and extracellular material appear normal. X 60,000

Figure 16: S. mutans 10449 incubated with the addition of SnF_2 (5 ppm F^-). Note frequency of electron dense deposits intracellularly (black arrows). Some cells do not appear round (white arrows) which is indicative of altered growth, and extracellular material appears altered. X 60,000



Methods and Materials

A stock culture of Streptococcus mutans NCTC 10449 was maintained by monthly transfer in thioglycollate medium supplemented with excess $CaCO_3$. For this experiment the culture was adapted to the complex medium of Jordan as described in the bacterial acid measurement phase of this contract. One ml. of the adapted culture was added to 6 flasks containing 200 ml. of Jordan's medium supplemented with 5 ppm F^- of either NaF, SnF_2, Na_2SnF_6, Na_2PO_3F or $SnCl_2$ (Sn^{++} = Sn^{++} in SnF_2) or H_2O. The flasks were incubated at 37° in a shaker bath for 2 days.

After day an aliquot of media and bacteria was removed for electron microscopic preparation. The specimens were fixed in 2.5% gluteraldihyde in 390 mos M phosphate buffer and postfixed in 1% osmium tetroxide in Veronal buffer (pH 7.3) (J. Histochem. Cytochem. 15:542-549, 1967). After dehydration in acetone and embedment in Spurr's epoxy medium (J. Ultrastruct. Res. 26:31-43, 1969), the resin was polymerized at 70° C.

Thin sections were prepared with a Reichertor LKB ultramicrotome using a diamond knife. Silver-gold-colored sections were either stained with aqueous uranyl acetate followed by lead citrate (J. Cell Biol. 25:407-408, 1965) or observed unstained at 90 kV with a Zeiss E M 10 electron microscope.

Results

Electron microscopy of the control specimens, i.e., bacteria incubated for 1 day in Jordan's media supplemented with 5% sucrose, typically demonstrated, well fixed coccal bacteria surrounded by extracellular polymers. The cells, many of which were in various stages of division, had characteristically intact cell walls and cell membranes. The bacterial cytoplasm was granular and electron dense bodies in the center of the cells was compatible with concentrations of DNA. Cell associated extracellular polymers were

abundant and morphologically appeared globular. No fuzzy coat was obvious on the cells (Fig. 13).

Specimens incubated in the same conditions yet with 5 ppm fluoride as NaF (Fig. 14) or 5 ppm tin as $SnCl_2$ (Fig. 15) morphologically appeared identical to the control specimens.

However, several growth alterations are suggested by the electron micrographs of those S. mutans subjected to 5 ppm fluoride on SnF_2. There is a suggestion of altered growth conditions since some cells deviate from being round. Also the extracellular material appears more granular than that seen in the other specimens (Fig. 16) Most striking, however, is the frequent appearance of electron dense bodies in the cells subjected to 5 ppm SnF_2 (Fig. 16) These electron dense areas, only found within the cells, are not staining artifacts since they are present before the cells are stained.

Discussion

The electron dense bodies found in those S. mutans specimens continuously incubated for 1 day in the presence of 5 ppm fluoride as SnF_2 appears similar to those structures noted in S. mutans cells which had 1 minute exposure, twice a day, to 250 ppm fluoride as SnF_2. Extrapolating from the electron probe results from the intermittent exposure trials, it appears evident that the electron dense bodies found in the SnF_2 group in these experiments is tin. The tin deposits appear to be concentrated greatly in the cells exposed to SnF_2; yet in those bacteria exposed to $SnCl_2$, there was no observations of the same electron dense bodies.

The SnF_2 molecule thus appears unique in depositing tin into the bacterial cells. Others have noted that fluoride is taken up and retained within bacterial cells (Scand. J. Dent. Res. 85:149-151, 1977; Arch. Oral Biol. 23:993-996, 1978). It is conceivable that the tin coupled with fluoride in the SnF_2 solutions is passively carried within the cell along with the fluoride and also becomes concentrated as noted with fluoride. Since chloride uptake into cells is different than fluoride, the variation between SnF_2 and $SnCl_2$ ultrastructurally and metabolically may be due to uptake and accumulation of tin only when coupled to fluoride.

The observation of tin in the bacterial cells exposed to only 5 ppm fluoride as SnF_2 appears to be a milestone in the concept of reduced plaque pathogenicity via slow release fluoride agents. First, the large accumulation of tin in those cells exposed to SnF_2 but not $SnCl_2$ gives strong, indirect evidence that fluorides, even in trace amounts, becomes concentrated

in the bacterial cells. The concept of fluoride concentrations within plaque bacterial cells enables explanation of the results found in previous experiments in which we found that even 0.5 ppm F^- alters metabolism and acid production in S. mutans. Secondly, the observation of tin storage within the cells may explain some of the subtle ultrastructural changes noted within the cells exposed to 5 ppm SnF_2 and the dramatic ultrastructural changes noted with intermittent exposure to higher concentrations of SnF_2. These structural findings noted with trace amounts of SnF_2 gave us optimism as to future positive findings with regard to alterations in bacterial growth and bacterial glucan experiments with low levels of fluoride which we are now conducting.

Atomic Absorption Experiments

Introduction

An *in vitro* antimicrobial testing system which had incorporated organisms grown on specimens and exposed intermittently to different treatment groups, had shown SnF_2 at 250 ppm to be the most effective agent. Electron micrographs of the organisms adherent to the enamel surfaces, which were exposed to all of the treatment groups, produced the finding of dense granular masses within the cells exposed to the SnF_2 at 100 and 250 ppm F^-. An electron microscope adapted to a microprobe accessory produced micrographs identifying tin as the main element contained within the granular masses. The purpose of this experiment is to observe the effects of SnF_2 at 100 and 250 ppm F^- on the acid production of preformed plaque and accumulating plaque and to show that only SnF_2 and not $SnCl_2$ can transport its Sn++ element into the cell and thereby causing possible toxic effects.

Materials and Methods

Microorganism

Streptococcus mutans is strongly implicated as a prime pathogen in the dental caries of rodents (J. Dent. Res. 39: 923-935, 1960), non-human primates (Caries Res. 3: 227-237, 1969) and man (Caries Res. 13: 190-200, 1969). This bacterium has only a small affinity for tooth surfaces, and its ability to colonize teeth is believed to be due to its rapid formation of polysaccharides after the initial adsorption rather than its affinity for tooth surfaces (Infect. Immun. 9: 624-630, 1974; Infect. Immun. 10: 1170-1179, 1974; Arch. Oral Biol. 20: 609-615; J. Dent. Res. 55: C226-C228, 1976). Sucrose appears to be necessary for the adherence of this organism to surfaces, yet *in vitro* evidence have shown this not to be necessarily true (Infect. Immun. 21: 1010-1019, 1978). *Strep. mutans* NCTC 10449, serotype c, was chosen for this experiment because of the epidemiology studies implicating this serotype to be the most prevalent in the human oral cavity (Odont. Revy. 23: 1-10, 1972).

Solutions

Pure cultures of *Strep. mutans* were maintained by transfer each month in fluid thioglycollate medium (Difco) supplemented with beef extract (20 v/v) and excess $CaCO_3$.

The growth medium for *Strep. mutans* consisted of broth (Jordon et al., 1960) supplemented with 50 mg/l $NaCO_3$ and 5% sucrose.

In vitro plaques were exposed to two tin compound solutions, 0.050% $SnCl_2$ and 0.041% SnF_2 (100 ppm F^-) with the Sn++ in $SnCl_2$ being equimolar to Sn++ in SnF_2. Plaques exposed to deionized H_2O were used as a baseline control. The

standard stock solution of inorganic tin at 1000 ug/ml concentration was diluted with 10% HCl and 8% citric acid as a stabilizer to obtain working standards of 10.0-0.5 ug/ml tin concentrations.

Plaque samples were digested readily with Soluene-350 at 20% (w/v) concentration with toluene for injection into the graphite furnace.

Experimental Design

Plaque formed in vitro

Strep. mutans were adapted and established in a log phase of growth before being inoculated into 3 groups of test tubes with 5 in each group containing Jordon's broth supplemented with 5% sucrose. 20 gauge nichrome wires were suspended separately in each tube 1 cm. from the bottom of the tube. The wires and tubes were vortexed and incubated at 37° C aerobically for a total of 2 days with intermittent exposures every 12 hrs. for 1 min. to separate tin compound solutions followed by an immediate 1 min. standing rinse in deionized H_2O. After the 2 days of growth, the adherent plaques were scraped off the wires with the inside of a platinum loop and deposited into tared conical centrifuge tubes containing deionized H_2O and spun at 4000 rpm for 25 min. to form a pellet. The supernatant was removed and the tubes with the pellets were allowed to dry in an 80° C oven for 3 days.

Acid production

To determine if the effect of the tin compound solutions differed between preformed plaques on wires, or plaques in the process of accumulating on wires (pilot study, J. M. Tanzer), pH readings were done on the broths which supported plaque growth for 12, 24, 36 and 48 hrs.

Atomic absorption

A Perkin-Elmer Model 407 atomic absorption instrument equipped with a

Perkin-Elmer Model HGA-2100 graphite furnace atomizer and its atomization control feature was used. A deuteruim discharge lamp was required to correlate the nonatomic absorption signal. A tin electrodelese discharge lamp was used for improved stability and sensitivity at the lower tin levels. To reduce noise, an electronic filter, Spectrum M-1021, was used. The unit has an amplification feature which extends the working range when needed. A Sargent SR recorder, which has a variable 1-10 mV input, was used for peak height readouts.

Nitrogen was used as the sheath and purge gas. After samples were introduced, the three-stage heating cycle was initiated. All absorption measurements were made in triplicate to confirm the reproducibility using peak height measurements.

Results

Acid production - experiments to be completed.

Atomic Absorption

Tin analysis using the graphite furnace has been reasonably successful but in this application of plaque material, we ran into problems. Comparing the graphite method to the EDL-AA (nitrous oxide) and the EDL-AA with background correction, we found the latter to be the most promising method (Figures 17, 18, and 19).

Figure 17: Graphite Furnace: background correction

Sample	Sn	Sample	Sn	Sample	Sn
11	ND*	6	0.097	11	0.13
2	ND*	7	0.065	12	ND*
3	0.032 µg	8	0.032	13	0.032
4	0.097	9	0.032	14	ND*
5	ND*	10	0.032	15	0.033

*ND=None Detected

Figure 18: EDL Scale Expansion--no background correction

Sample	Sn	Sample	Sn	Sample	Sn
1	0.8 µg	6	0.48	11	2.8
2	1.2	7	1.04	12	1.9
3	2.2	8	2.2	13	2.0
4	1.4	9	1.04	14	2.2
5	1.8	10	1.8	15	2.9

Figure 19: EDL Scale Expansion--background correction

Sample	Sn	Sample	Sn	Sample	Sn
1	ND*	6	ND*	11	1.34
2	ND*	7	1.04 µg	12	1.56
3	ND*	8	1.49	13	1.30
4	ND*	9	1.04	14	1.11
5	ND*	10	1.30	15	1.41

*ND=None Detected

Discussion

An investigation was directed toward the possible entry of the Sn++ ion via the fluoride or chloride group into concentrated areas either in the plaque matrix or transported actively or passively into the cell.

The electron microscopy and microprobe results show that tin, either bound or in the free ionic state, does enter the cell and accumulate into granular masses. It is to be seen whether or not there exists a critical point of tin accumulation causing cellular toxic effects upon the metabolism processes adding to the effect that fluoride ions already have upon the glycolitic cycle and the transport of glucose through the cell membrane.

Pilot studies have shown a difference in preformed plaques compared to accumulating plaques on the rate of acid production observed in an *in vitro* system. A greater reduction of acid production was believed to have been seen in the accumulating plaque growth intermittantly exposed to SnF_2 as shown by the pH indicator bromcresol purple. Our more sophisticated and simplified method should resolve why frequent contact with SnF_2 upon growing cells may be more effective than one exposure where only the surface bacteria are affected.

Technically, the atomic absorption method needs to be further developed and modified. As shown in the results, even the method of addition with the graphite furnace was inconsistent. It appears the tin peak signal is being somehow suppressed in the samples.

However, once the method is perfected, we will be able to clarify our electron microscopy and microprobe results. Once plaque matrix and the bacterial cells are separated by centrifugation techniques, samples analyzed by AA may show Sn++ from $SnCl_2$ present in the plaque matrix and Sn++ from SnF_2 present within the cells.

SLOW RELEASE OF FLUORIDE FROM TEMPORARY RESTORATIVE MATERIAL

Introduction

In preparation for the hamster study, a series of experiments are presently being conducted to determine various formulations of the selected fluoride compound derived from the in vitro studies (experimental data supports the choice of SnF_2) so that a concentration of fluoride in the temporary restoration is found to release at least 1 ppm F^-/da./30 da. This concentration of F^- will then be used for all subsequent hamster studies. The relative ratio of IRM/ oral cavity area of the hamster is calculated to be proportional to that of a human subject.

Materials and Methods

In the trials to date, circular holes 16.0 mm^2 by 5 mm in depth is prepared on the buccal surface of 4 human molars. Intermediate Restorative Material (Caulk IRM®) mixed with 30% and 50% SnF_2 (weight F^-/weight IRM powder) was used for the restoration material of 2 of the teeth. The other 2 teeth had zinc oxide-eugenol (ZOE) mixed with 30% and 50% SnF_2 to fill the cavities. Each tooth was then placed in one liter of normal saline (0.85% NaCl) which simulated the volume of saliva secreted per day since the average salivary secretions are estimated at approximately 800 ml/day (J. Dent. Res. 53:246-266, 1974). The flasks were then placed in a water bath shaker at 37° and agitated at a slow speed setting. After each 24 hours, a 1 ml aliquot was removed from each of the four flasks and stored for fluoride analysis. The solution was replaced after each day for each test specimen. These procedures were repeated

for 30 days with samples collected every other day until the fluoride had depleted from the temporary restorations as shown by intermittent fluoride analysis. The fluoride analysis for each sample was performed by specific fluoride ion electrode measurements.

Results

In all cases IRM released all fluorides after 10 days of exposure to saline solutions. The ZOE material showed greater promise in having extended the release process to 25 days before F^- extinction. However, the F^- levels after 12 days (0.35 ppm F^-) may be below therapeutic effects required for actual field combat use. See Figure 20.

Assuming the F^- may have extinguished only from the surface layers of the temporary restoration, a repolishing of the surface was attempted to show if fluoride was still present below the surface. However, no increase of the fluoride release was found after the resurfacing was completed as shown by Table 1.

An experimental 80% F^-/ZOE was found to lack a firm support base for the SnF_2 crystals within the cavity of the tooth. Future experiments are being conducted to find the right amount of releasing factors coupled with a long lasting restoration.

Discussion

This preliminary investigation utilized two common temporary restorative materials available to most dental clinicians. IRM is a plastic reinforced zinc oxide-eugenol composition used for temporary restorations lasting up to one year. It has greater strength qualities than the pure zinc oxide-eugenol material; however, the amount of F^- released from the IRM restoration is too rapid resulting in the depletion of SnF_2 crystals as shown in Figure 20.

Current research points to the 50% SnF_2/ZOE as the most promising combination for future studies. Materials testing to determine hardness of the combinations of restorative material will be completed before they are utilized in the hamster studies.

Other experimental temporary restorative materials will be investigated if these two widely used materials appear to be unsatisfactory for future *in vivo* hamster studies.

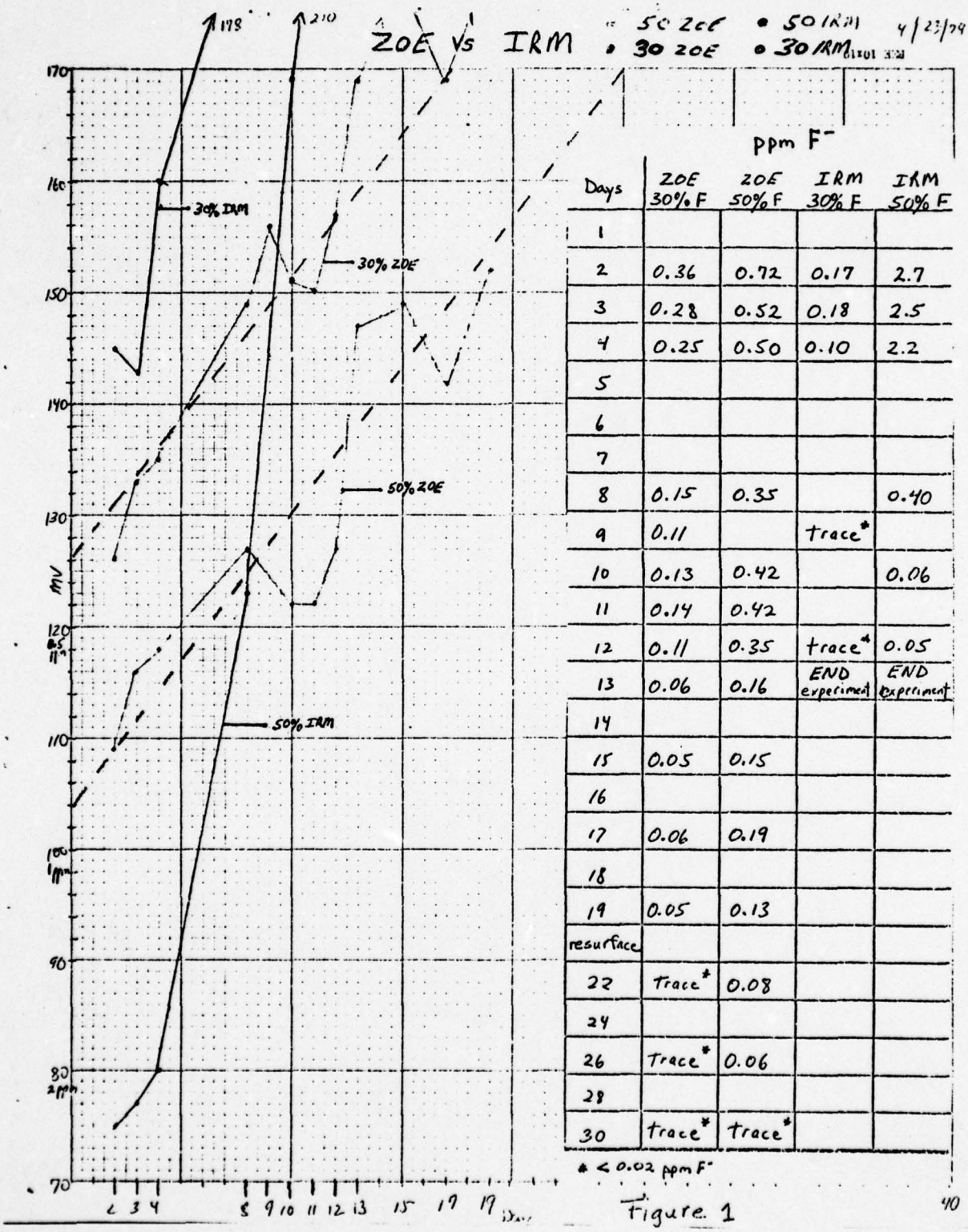


Figure 20. The concentration of fluoride ions released from 4 experimental temporary restorations incorporating SnF_2 as the active ingredient. The duration of the study lasted 20 days with 1 liter of normal saline replaced after each day for each restoration. Fluoride electrode measurements were performed on the days shown in table format.

Appendix A

Communications With Naval Dental Research Institute

DEPARTMENT OF THE NAVY
NAVAL DENTAL RESEARCH INSTITUTE
NAVAL BASE
GREAT LAKES, ILLINOIS 60088

IN REPLY REFER TO:
NDRI-43-tg
5600
03 April 1979

Dr. J. Hock
University of Connecticut Health Center
School of Dental Medicine
Farmington, Connecticut 06032

Dear Dr. Hock:

Please forward the text of the paper you presented at the recent IADR meeting entitled "Clinical Trial to Test Antiplaque Effect of SnF2 Mouthrinse".

We at the Naval Dental Research Institute (NDRI) are using topical SnF2 preparations as antimicrobial agents and would be most interested in knowing the details of your latest study.

Thank you.

Sincerely yours,

J. C. CECIL III DMD, MPH
LCDR DC USN
Chief, Dental Care Delivery Division

THE UNIVERSITY OF CONNECTICUT
HEALTH CENTER

April 10, 1979

J. C. Cecil III DMD, MPH
LCDR DC USN
Chief, Dental Care Delivery Division
Department of the Navy
Naval Dental Research Institute
Naval Base
Great Lakes, Illinois 60088

Dear Dr. Cecil:

Dr. Hock forwarded your letter to me since I have been involved in the antiplaque studies of SnF_2 for some time and since I was principal investigator of the SnF_2 clinical trial.

I am enclosing the paper recently submitted to J. Clinical Perio. which concerns the clinical study reported at I.A.D.R. Because we have not heard whether this paper has been accepted as of yet, I hope you will not widely distribute this manuscript.

These studies and others on the antiplaque effects of SnF_2 have been supported by and performed in conjunction with the U.S. Army Institute of Dental Research. Because of the mutual interests we share with regard to preventive dentistry for military personnel, I hope you can tell us how you are using SnF_2 clinically, and how much or if any success you have noticed with this agent in a clinical setting. I am most interested in hearing from you.

Also, I have just completed a review entitled "Current Status of SnF_2 as an Antiplaque Agent" which has been accepted for publication in Pediatric Dentistry. If you would like a copy of this manuscript, please let me know.

Best regards,

Norman Tinanoff

Norman Tinanoff, D.D.S., M.S.
Assistant Professor
Department of Pediatric Dentistry

NT/i

enc.

DENTAL MEDICINE
FARMINGTON CONNECTICUT 06032

Appendix B

List of Publications and Presentations in 1979
Supported by U.S. Army Contracts DAMD 17-77-C-7058
and DAMD 17-78-C-8066

Papers published:

1. Tinanoff, N. and Tanzer, J.M. Bacterial Pellicle-Like Substances and Polyphosphates Formation by Enamel-Adherent Oral Microorganisms. Pediatric Dentistry 1:1-6, 1979.

Paper in press:

1. Tinanoff, N. and Weeks, D. Current Status of Fluorides as an Antiplaque Agent. Pediatric Dentistry (September 1979).

2. Tinanoff, N., Hock, J., Camosci, D. A. and Hellden, L. The effect of Stannous Fluoride Mouthrinse on Dental Plaque Formation. J. Clin. Perio., 1980.

Papers in preparation:

1. Tinanoff, N. and Camosci, D.A. Microbiologic and Electron Microscopic Studies of the Antiplaque Effects of Fluorides in vitro Arch. Oral Biol.

2. Tinanoff, N. and Camosci, D.A. Fluoride Update for Connecticut Residents. J. Conn. St. Dent. Assoc.

3. Camosci, D.A. and Tinanoff, N. Competitive Attachment Between Streptococcus mutans and Streptococcus sanguis in Glucose and Sucrose Media. J. Dent. Res.

Presentations:

1. Tinanoff, N. Camosci, D.A. and Gross, A. Intermittent Exposure of fluorides on Bacterial Colonization of Enamel In Vitro. Proc. 57th Ann. Meeting, Int. Assoc. Dent. Res., New Orleans, 1979.

2. Tinanoff, N. and Tanzer, M.M. Electron Microscopy of Pellicle Formed by Enamel-Adherent Organisms. Proc. 57th Ann. Meeting Int. Assoc. Dent. Res., New Orleans, 1979.

3. Tinanoff, N., Hock, J., Camosci, D. and Hellden, L. Clinical Trial to Test Antiplaque Effect of SnF_2 Mouthrinse. Proc. 57th Ann. Meeting Int. Assoc. Dent. Res., New Orleans, 1979.

4. Camosci, D.A., Tinanoff, N. and Gross, A. Intermittent Exposure of Fluorides and Bacterial Colonization of Enamel In Vitro. Am. Soc. Microbiol., Conn. Valley Branch, Storrs, CT, 1979.

Appendix C

Abstracts of Papers Presented in 1979

FORM A

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH
and
AMERICAN ASSOCIATION FOR DENTAL RESEARCH

ABSTRACTS MUST BE RECEIVED BY OCTOBER 16, 1978

Complete: Items 1—5 below and type abstract within box, following instructions on reverse side.
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Mr. David Camosci
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Farmington, CT 06032

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Intermittent exposure of fluorides on bacterial colonization of enamel in vitro.
N. TINANOFF, D. CAMOSCI*, A. GROSS, U. of Conn. Health Center, Farmington, CT and U.S. Army Inst. Dent. Res., Washington.

An in vitro model was used to observe the effect of intermittent exposure of various fluorides on colonization of (1) S. mutans and (2) S. sanguis on enamel. All but the outer surface of 24 enamel cylinders was coated with wax. These specimens were then suspended by a nylon line in Jordan's broth containing 5% sucrose, and inoculated with either S. mutans 10449 or S. sanguis 10558. Twice daily the cylinders were removed from the medium and exposed 1 min. to one of the following agents: H20; NaF(100ppmF$^-$); SnF_2(100 ppmF$^-$); SnF_2(250ppmF$^-$); $SnCl_2$(Sn equals Sn in 100ppm SnF_2); and Na_2SnF_6(100ppmF$^-$). After exposure the cylinders were rinsed in phos.buffer and replaced in the growth medium. At 48 hr. the wax was removed from the cylinders so that only the adherent bacteria on the surface enamel remained. Quantitation of the enamel-adherent bacteria of the 4 cylinders in each group was performed using spread plate counts and spectrophotometric techniques. For the S. mutans trial only SnF_2 significantly reduced the number of bacteria on enamel with 72% and 99% decrease respectively for the 100 and 250 ppm SnF_2 group. For the S. sanguis trial the decrease was comparable with 48% and 97% reduction noted in the SnF_2 group. Since NaF and $SnCl_2$ showed little effect on bacterial colonization, the large reduction noted for SnF_2 cannot be explained by Sn^{++} or F^- alone. Furthermore, the effectiveness of SnF_2 in reducing bacterial colonization on enamel appears to increase at higher concentrations.

Supported by U.S.Army Contract DAMD-17-77-C-7058

6. Reviewer's Rating:	7. Disposition:
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Farmington, CT 06032

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Clinical trial to test antiplaque effect of SnF_2 mouthrinse. P. TINAKOFF, J. HOCK*, D. CAMOSCI and L. MELLOCH, Sch. of Dent. Med., Univ. of Conn. Health Center, Farmington, CT

The efficacy of SnF_2(250 ppm F^-) mouthrinse as an antiplaque agent was tested in a double blind, cross-over design study involving 27 dental students. For 5 days the subjects refrained from mechanical oral hygiene, and rinsed under supervision twice a day for 1 min with either SnF_2 or placebo. The regimen was repeated with the alternate rinse the following week. Mean G.I.; plaque score (PS)(Martins and Meskin,1972); plaque wet weight (PWW); colony forming units (cfu/mg) and total colony forming units (Total CFU) were calculated for 6 teeth (Radford,1955) from each subject. Plaque wet weight and bacterial analyses were performed on the organic deposits removed from the supragingival area of the 6 teeth. Pearson r and correlative analyses were performed.

The SnF_2 rinse produced the following % reductions in the parameters tested: G.I.-17% (N.S.); PS-8% (p<.10); PWW-31% (p<.01); CFU/mg-30% (p<.01); Total CFU-50% (p<.01). Correlation analyses showed that Total CFU was the best parameter to judge efficacy. The low correlation between PS and Total CFU in the SnF_2 group suggest that visual deposits on the teeth were not all bacterial. Due to the apparent antiplaque properties of SnF_2, as well as its known value in caries prevention are known, this agent appears to be a practical candidate as a preventive agent. Longer term studies are necessary to delineate its value in reducing gingivitis.

Research supported by U.S. Army Contract #DAMD17-77-C-7058.

6. Reviewer's No.					7. Decision				
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Dr. Norman Tinanoff
Department of Pediatric Dentistry
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Farmington, Connecticut 06032

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Electron Microscopy of Pellicle Formed by Enamel-adherent Organisms. N. TINANOFF* and J. M. TANZER, Univ. of Conn. Health Center, Farmington, Connecticut

We have previously described some of the parameters involved in early plaque formation by Streptococcus mutans on enamel in vitro (Infec. Immun. 21:1010-1019, 1978). The purpose of this investigation was to observe visually and electron microscopically the characteristic features of the attachment of other species to enamel. Wire mounted enamel specimens which had been thoroughly pumice cleaned were suspended in Jordan's medium supplemented with 5% sucrose and inoculated with one of 15 strains, including S. sanguis, S. salivarius, S. mutans, S. faecalis, Actinomyces viscosus and A. naeslundii. After visual observation, enamel adherent representative specimens were processed for electron microscopy and sections of the bacteria-enamel interface were prepared.

Adherent bacterial masses were evident on enamel with all S. mutans, S. sanguis and Actinomyces sp. tested, were variably evident with representatives of S. salivarius, and were not found with S. faecalis. Ultrastructurally bacterial attachment to enamel appeared to be mediated by a bacterially produced 'pellicle' which covered the enamel surface. This structure could not have been salivary derived since no saliva or exogenous glycoproteins were added to the incubations. The ability of bacteria to produce this extracellular polymer may be a critical determinant of initial stages of bacterial attachment to enamel.

Supported by U. S. Army Contract DAMD17-77-C-7058 and NIDR Grant DE-03758-03.

6. Reviewer's Rating:					7. Disposition:				
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Appendix D

Copy of Paper "In Press" to Pediatric Dentistry

CURRENT STATUS OF SnF_2 AS AN ANTIPLAQUE AGENT

By

Norman Tinanoff, D.D.S, M.S.

and

Donald B. Weeks, D.M.D

School of Dental Medicine
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Farmington, Connecticut 06032

Acknowledgement: This review was supported by U.S. Army Contract #DAMD 17-78-C-8066

Communications to: Dr. N. Tinanoff
Department of Pediatric Dentistry
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Farmington, Connecticut 06032

Although it is evident that fluorides are very effective in reducing dental caries, the manner in which these compounds accomplish this is still not fully understood. The concept that fluoride decreases the solubility of enamel through the formation of fluorapatite, thus protecting against bacterial acid production, has long been accepted. What defies explanation is how such small amounts of fluorapatite formed by the enamel-fluoride interaction can account for the relatively large degree of caries reduction which occur either from topical or systemic fluorides.

The apparent paradox has spurred investigations into possible antimicrobial mechanisms which could explain how fluoride reduces caries. The purpose of this review is to describe the findings concerning the plaque altering properties of fluoride, and specifically stannous fluoride, which have recently been noted to have important antiplaque properties.

The effect of sodium fluoride on bacterial metabolism has been known for some time and is relatively well understood. Inhibition of acid production by salivary and plaque bacteria in vitro has been demonstrated with less that 1 ppm F^- [1,2]. Plaque collected from subjects living in fluoridated areas, furthermore, has shown smaller increases in acid production with sucrose than plaque from subjects living in non-fluoridated areas[3]. These findings may be explained by the observation that fluoride alters the bacterial enzyme, enolase, which is essential for the degradation of simple sugars to lactic acid and is also essential for the transport of sugars across the bacterial cell membrane[4]. The inactivation of enolase is the result of fluoride binding with the magnesium component of this enzyme[5]. Fluoride ions acting in this manner could reduce bacterial acid production and might account for some of the caries inhibition noted for this agent.

While inhibition of acid production by fluoride is not controversial, there is less clear evidence concerning ability of sodium fluoride to reduce

the quantity of plaque. Decreases in the amount of plaque polysaccharide have been reported *in vitro* with 10 ppm F^- and 70 ppm F^- as NaF [6,7], yet *in vivo* studies showing the effect of fluoride on the quantity of plaque have provided only modest results. Plaque collected from subjects living in optimally fluoridated or highly fluoridated areas have been found to contain slightly less extracellular polysaccharide or slightly less visual plaque, respectively, compared to plaques collected from areas deficient in fluoride [8, 9]. Birkland has also shown that a weekly rinse of 0.2% NaF (1,000 ppm F^-) produced a small but significant reduction in plaque dry weight [10]. However, intensive fluoride therapy, i.e., frequent application of concentrated solutions of NaF have provided better results. Loesche, et al., besides demonstrating lower plaque and gingivitis scores in subjects frequently applying 1.23% acidulated phosphate fluoride topically, also showed that the number of *Streptococcus mutans* relative to *S. sanguis* in plaque was also lowered [11, 12].

The earliest reference to SnF_2 having an effect on oral flora was that reported by Lilienthal in 1956. He found that 0.01% SnF_2 (25 ppm F^-) inhibited acid formation by saliva and salivary sediments *in vitro* [13]. Dramatic plaque reducing properties of stannous fluoride were later observed in 1959 when König noted plaque inhibition in rats when 0.1% SnF_2 (250 ppm F^-) was applied once a day for 35 days to rat molars [14]. SnF_2 was again noted in 1976 to reduce the number of bacteria adherent to enamel *in vivo* as observed by electron microscopy. Enamel cylinders, embedded in a maxillary Hawley appliance were worn by one subject for 2 or 7 days while performing various mouthrinse regimens. Since stannous fluoride reduced bacterial colonization on enamel, but sodium fluoride did not, the antiplaque effect found in this limited study was believed not solely due to the fluoride ion [15]. Subsequently, rats inoculated with *Actinomyces viscosus* and *Streptococcus mutans* were reported to

have a 71% reduction in plaque when treated with SnF_2 [16].

Recent studies have definitely established that SnF_2 affects the oral flora in man. Subjects using SnF_2 mouthrinse have demonstrated a reduction of bacterial acid production in salivary samples [17] and in intact dental plaque [18]. SnF_2 at high concentrations (1,250 ppm F^-) used daily as a mouthrinse was also found to decrease by 99% the number of bacteria per ml of saliva, while NaF at the same concentration had little influence on salivary bacteria [19]. Other studies have revealed between 74-99% reduction in the number of microorganisms in dental plaque from subjects using relatively dilute SnF_2 mouthrinse twice daily [20, 21].

The ability of SnF_2 to reduce plaque formation has been impressively demonstrated in several clinical trials. One single application of 8% SnF_2 was found to reduce plaque weight and visual plaque scores on 27 children [22]. Another clinical study has found that toothpastes which contain SnF_2 have anti-plaque properties [23]. In an experimental series in which 12 subjects used 0.2 or 0.3% SnF_2 mouthrinse twice daily for 4 days, plaque inhibition of SnF_2 was comparable to that of chlorhexidine. In a second experiment in the same report, 5 students who discontinued oral hygiene for 3 weeks rinsed with sucrose for 1 week to augment plaque formation, and then rinsed with SnF_2 for 2 weeks. The mean Plaque Index score was a low 0.24 for this group after the third week [24]. Another clinical trial on 27 subjects who used 0.1% SnF_2 or a placebo mouthrinse twice a day for 5 days has shown significant reductions in visual plaque score, plaque wet weight, number of bacteria per mg plaque, and total number of bacteria collected from 6 representative teeth. In this study, the total number of bacteria was considered to be the best index for plaque reduction, and SnF_2 mouthrinses reduced plaque by 50% using this criterion [25]. Subsequent studies using the same experimental design and the same plaque indices, however, have found that 0.1% SnF_2 was not nearly as effective as 0.2% chlorhexidine

when these agents were compared in a twice daily mouthrinse regimen [26].

The mechanism responsible for alteration of plaque formation by SnF_2 are, as yet, not well understood. Some information on the way SnF_2 reduces plaque has been derived from electron microscopic observations of the enamel cylinders worn *in vivo* during mouthrinse procedures. When SnF_2 was used as a mouthrinse once daily for 2 days, the number of bacteria on the enamel appeared greatly reduced; when SnF_2 was used twice daily, the bacterial colonization was essentially eliminated (Figure 1). Rinsing for 7 days with SnF_2 produced a thick amorphous pellicle on the enamel with the bacteria generally appearing as a non-aggregated layer on the enamel (Figure 2). Based on these electron microscopic observations, it has been postulated that the variation in colonization noted with SnF_2 may be due in part to altered adhesion of bacteria to enamel or altered cohesion of bacteria [15].

Some authors have suggested that the tin component of SnF_2 may be responsible for the antiplaque properties. The stannous ion could conceivably compete with calcium for acidic groups on bacterial surfaces or acidic groups on teeth and thus inhibit plaque formation [24, 27]. There is also some evidence that a cell wall component in gram positive bacteria, lipoteichoic acid, may be the "glue" which binds bacteria to tooth surfaces [28, 29], and divalent cations (i.e., Sn^{++}) may possibly interact with this highly negatively charged polymer, thereby changing the surface potential of bacteria [23, 30]. Demonstration of the divalent cation effect was noted in a clinical study in which mouthrinses comprised of either aluminum, zinc, and magnesium or stannous salts reduced plaque formation [31]. However, divalent cations cannot entirely explain the substantial antiplaque properties of SnF_2. Stannous chloride mouthrinse equimolar to SnF_2 was noted to have some effect on plaque in the *in vivo* plaque model system previously mentioned, but the reduction in plaque was not as dramatic as that found with SnF_2 [15]. Other studies have also observed that

$SnCl_2$ is not as effective as SnF_2 in reducing the amount of plaque which adheres to enamel *in vitro* [32], or *in vivo* [31], or in inhibiting pH changes in dental plaque [33]. One hypothesis mentioned for the decreased effectiveness of $SnCl_2$ is that it rapidly hydrolyses in water [33]. It is also possible that since fluoride is known to become bound to plaque bacteria [34], as well as to enamel [35], the fluoride ions in SnF_2 may enhance the retention of tin in plaque and thereby make this agent more effective as an antiplaque agent.

The possibility that SnF_2 may selectively depress specific organisms responsible for caries formation has also been examined in reports by Keene, et al. [36, 37]. Interproximal tooth sites in humans were tested for presence or absence of *Streptococcus mutans* after four days of twice daily flossing in conjunction with topical application of 10% SnF_2 (24,000 ppm F^-) or saline. It was noted that there was a significantly greater change of positive *S. mutans* sites to negative *S. mutans* sites in the SnF_2 treated group [36]. The results reported, however, may not truly represent a selective action against *S. mutans* but rather a generalized plaque depression, since other studies have not shown any change in the ratios of organisms due to SnF_2 [20, 21].

To date, the studies that have examined the plaque inhibitory effect of SnF_2 have been too short in duration to observe changes in gingival health in humans [25, 38] and a question still remains as to the effect of topical SnF_2 on gingivitis. Two studies in dogs have shown reductions in gingival inflammation with intermittent SnF_2 rinses [39, 40]. Furthermore, SnF_2 has been reported to be the most effective fluoride agent against some known periodontopathic microorganisms [41]. Clinical trials on human populations utilizing the experimental gingivitis models need to be conducted. If such trials demonstrate that SnF_2 reduces gingivitis, then further studies should be undertaken to examine long term effects of SnF_2 on the development of periodontal disease. Long term studies also need to be performed to observe changes in the oral flora and any

possible side effects caused by the drug. The use of disclosing dyes to visualize plaque area, however, does not appear appropriate in studies with SnF_2 since non-bacterial deposits (Figure 3) accumulate readily when teeth are exposed to SnF_2 and the deposits may look similar to plaque [15, 42].

Based on its demonstrated plaque inhibition, SnF_2 may be useful for suppression of plaque following periodontal surgery. The advantages of maintaining oral surgical sites plaque-free have been documented [43, 44] and the ability of one antiplaque agent (chlorhexidine) to improve periodontal surgical results has been well established [43, 45]. Comprehensive clincial trials are needed to document the clinical impression that SnF_2 rinses improve healing following periodontal surgery.

Since clinical usage of SnF_2 preceded the strict drug guidelines now in effect in the U.S., this agent has not gone through rigorous trials to establish safety. However, the only reported side effect in its many years of use is staining of teeth (Figure 4). This stain, however, is said to be less tenacious than that attributed to chlorhexidine and in most cases is easily removed by a professional prophylaxis [24]. There is some objectionable taste associated with SnF_2; yet, when flavored commercial products are diluted to mouthrinse concentrations, the astringent metallic taste is minimal. Also important to remember is that SnF_2 has poor stability as an aqueous solution and therefore should be used soon after it is mixed with water. Commercial products most often have a glycerine base and consequently have an indefinite shelf life before they are diluted with water.

It is well established that SnF_2, as well as other fluoride agents, is effective in caries prevention. The development of a mouthrinse capable of reducing plaque formation, while at the same time increasing the resistance of teeth to demineralization, may constitute an important advance in prevention of both caries and periodontal disease.

Figure Legends

1. Scanning electron micrographs from surface enamel placed in a Hawley appliance and worn in one subject's mouth for 2 days. Amorphous pellicle, as well as reduced deposits of bacteria are present when SnF_2 (100 ppm F^-) is used as a mouthrinse once a day (A). When SnF_2 was used twice a day, pellicle covers the enamel, but no bacteria are apparent.
2. Scanning electron micrograph of enamel worn in the mouth for 7 days using SnF_2 mouthrinse twice a day. Only a monolayer of non-aggregated coccal bacteria is evident.
3. Transmission electron micrograph of enamel worn in the mouth for 7 days with SnF_2 mouthrinsing twice a day. A thick laminated deposit (pellicle) is found on the enamel surface (arrow).
4. Subject who rinsed twice a day for 6 months with SnF_2 (100 ppm F^-). Note stain on proximal, cervical tooth surfaces and around anterior restorations.

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Appendix E

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MICROBIOLOGICAL, ULTRASTRUCTURAL AND CHEMICAL ANALYSES OF THE ANTIPLAQUE PROPERTIES OF FLUORIDE COMPOUNDS IN VITRO

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Running Title: Antiplaque properties of fluoride

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Summary

The growth of bacteria on enamel *in vitro* was used to investigate the effect on plaque formation of 1 min., twice daily exposure to various fluoride treatments. After 2 days, microbial quantitation of the number of adherent, *S. mutans*, *S. sanguis* or *A. viscosus* cells showed that in all cases, SnF_2 at 100 or 250 ppm F^- significantly reduce the bacteria; whereas, NaF (100 ppm F^-), $SnCl_2$ (Sn++ equimolar to 100 ppm SnF_2) and Na_2SnF_6 (100 ppm F^-) produced no significant change. Electron microscopy of the *S. mutans* specimens exposed to SnF_2 (250 ppm) revealed bacterial separation from the enamel and several morphological alterations of the cells themselves. Electron dense granules, identified as tin by the electron microprobe, were also noted in the bacteria exposed to SnF_2. Atomic absorption spectrophotometry of similarly processed specimens revealed high levels of tin in plaque samples treated with SnF_2 but not with $SnCl_2$. These findings support the view that SnF_2 has antiplaque properties and that the mechanism its action may be due to the accumulation of tin in the bacteria.

Introduction

It is now widely accepted that the etiology of both dental caries and periodontal disease is associated with the accumulation of bacterial plaque on teeth. With regard to dental caries, fluoride as NaF has been shown to alter the pathogenicity of the dental plaque as well as reacting physiochemically with hydroxyapatite to reduce enamel solubility (Larson et al., 1976). The reduced pathogenicity may be due in part to reduced bacterial metabolism. Fluoride ions, even at 1 ppm, have been shown to alter bacterial metabolism by interfering with transport and utilization of carbohydrates with a consequent reduction of bacterial acid production (Hamilton, 1977). Clinical studies, though, have not clearly shown reduction of bacterial numbers on teeth by NaF except when applied at high concentrations (Loesche, Syed, Murray, Mellberg, 1975).

Recently, human clinical trials (Svatun et al., 1976; Tinanoff et al., 1980), animal studies (König, 1959; Hock and Tinanoff, 1979), and an in vivo plaque model (Tinanoff et al., 1976) have shown that SnF_2 mouthrinses markedly reduce the quantity of bacteria on teeth. The differences between NaF and SnF_2 in altering plaque formation may be explained by the divalent cation, Sn++, in which is thought to interfere with bacterial adhesion and/or cohesion (Skjorland, Germo and Rölla, 1978). Yet divalent cations themselves cannot completely explain the antiplaque effect of SnF_2, since $SnCl_2$ appears less effective than SnF_2 as an antiplaque agent (Tinanoff et al., 1976; Svatun and Attramadal, 1978).

The purposes of the present in vitro experiments were to: (1) quantitate the alteration of colonization of several plaque forming microorganisms on

enamel substrata in response to intermittent exposures to various fluorides compounds, and (2) to further explore the antiplaque mechanisms of these agents through ultrastructural and chemical analyses.

MATERIALS AND METHODS

Enamel Preparation

For the microbiological studies, enamel cylinders, 24 mm^2, were prepared from the smooth surfaces of tap water-stored human anterior teeth using a diamond circular drill (Pfingst & Co., New York, NY). The enamel cylinders were polished with a slurry of pumice to remove organic material, washed in deionized water using an ultrasonic cleaner, and then autoclaved. These cylinders were coated with dental wax (inlay casting wax, Kerr products, Emeryville, CO) to cover all areas of the cylinders except for the enamel surface. A nylon filament which suspended the cylinders in test tubes was attached to the wax orientating the enamel surface vertically. The nylon filament was then supported by a cotton plugged hole in a rubber stopper. Prior to experiments, the enamel cylinders with attached nylon filaments were placed in 70% ETOH for 15 min. to disinfect the surfaces from microbial contamination during the wax preparation. The cylinders were then rinsed with water to remove the residual alcohol.

For electron microscopy, extracted human molars were cut tangentially with a carborundum disc to make approximate 40 mm^2 enamel specimens. A hole to accept a 20 gauge nichrome wire was made through the specimens with a dental bur. The specimens were also polished with pumice and thoroughly washed. Specimens were suspended by wire, placed in capped test tubes, and autoclaved.

Fluoride Compounds and Controls

The following fluoride solutions, at a concentration of 100 ppm F^-, were tested to determine their ability to alter the colonization of bacteria on enamel *in vitro*: NaF (0.022% w/v, pH 5.3), SnF_2 (0.041%, pH 3.8), and Na_2SnF_6 (0.024%, pH 3.5). Pilot studies had shown that SnF_2 had greater antiplaque effect at higher concentrations; therefore, a solution of 250 ppm F^- as SnF_2

(0.10%, pH 3.7) was also tested. $SnCl_2$, 0.05%, (pH 2.9) and $SnCl_2$, 0.15% (pH 2.8), equimolar to Sn++ in SnF_2 100 ppm F^- and 250 ppm F^- respectively, were used to compare the possible tin ion effect. Deionized water (pH 6.6) was the control to which all the solutions were compared.

Microorganisms and Media

A streptomycin-resistant mutant of Streptococcus mutans NCTC 10449, Bratthall serotype c, known to produce heavy plaque in vitro (Tinanoff, Tanzer and Freedman, 1978), to be cariogenic in rats (Tanzer, J. M., 1979) and to be representative of the most frequently found human serotype in Europe and in the U.S. (Bratthall, 1972; Shklair and Keene, 1973), was used in this study. Streptococcus sanguis ATCC 10558, a species found in high numbers in the human oral cavity and considered non-cariogenic (Fitzgerald, 1963; Fitzgerald, 1968) and Actinomyces viscosus M100, suspected as a contributor to root surface caries and gingival inflammation (Jordan and Hammond, 1972) were also tested in this study.

Stock cultures were maintained by monthly transfer in fluid thioglycollate medium (Difco) supplemented with meat extract (20% v/v) and excess $CaCO_3$. For the experiments, cultures were adapted to growth in the complex medium of Jordan et al. (1960), supplemented with 50 mg of Na_2CO_3/l and containing 5% sucrose. Dilutions and sonication broths consisted of a 0.01M phosphate buffer (pH 7.0) containing 0.05% yeast extract (YE:BBL). The medium used for the enumeration of the microorganisms was mitis-salivarius agar (MS: Difco) containing 0.01% potassium tellurite.

Experimental Design

Four prepared enamel specimens were suspended separately in each of the six fluoride or control solutions for 1 min. (Figure 1). The enamel specimens were individually rinsed in 100 ml of water (deionized) for 1 min. to reduce any carry-over of the fluoride solutions into the growth medium and then

suspended in test tubes containing 10 ml of Jordan's medium containing 5% sucrose. The tubes were then inoculated with 0.2 ml of a sucrose-adapted log phase culture of the microorganisms to be studied. After 12 hours of incubation at 37°, the enamel surfaces were again exposed to the same fluoride solutions or controls for 1 min. and then rinsed for 1 min. in 0.01M phosphate buffer before being returned to the growth medium. Exposure to the test solutions was repeated every 12 hours to simulate a twice daily mouthrinsing regimen (Figure 1). The enamel specimens were transferred to fresh medium after the second exposure to the fluoride solutions (24 hours). At the end of 48 hours the enamel specimens with adherent plaque were either processed for electron microscopy, or the adherent microbial mass on each cylinder was quantitated by microbial enumeration and optical density.

Microbial Enumeration

The wax was meticulously removed from all areas of the enamel cylinders using a sterile scalpel, and then only the specimens with microorganisms on the surface enamel were placed into 2 ml of YE broth. The enamel and adherent bacteria were sonicated (Bronson, Model W185) for 25 seconds using a microprobe tip and set at 50 watts with the output at 4. This procedure separates the plaque from the enamel and disrupts bacterial chains (Liljemark and Schauer, 1977). Immediately, 1.0 ml of the sonicated samples were serially diluted and 0.1 ml from the 10^{-5}, 10^{-6}, and 10^{-7} dilutions were spread in duplicate on MS agar. All plates were incubated for 2 days in an enriched CO_2 environment (candle jar) at 35°. Plates containing 30 - 300 CFU were counted with the aid of a darkfield colony counter.

Spectrophotometric Readings

Optical density readings were used to correlate the amount of plaque found on the enamel surface with that of the bacterial spread plate counts. To each

previously undiluted sample 1.0 ml of YE broth was added and sonicated again for 25 seconds followed by O.D. readings at 600 nm.

Electron Microscopy

The two phases of the experiment, 1) the plaque growth of S. mutans NCTC 10449 and 2) the intermittent exposure to the test agents were repeated identically for the electron microscopic phase of the experiment. Only the technique of enamel specimen suspension in the test tubes was altered to eliminate wax interference with dehydration and embedment.

At 48 hours, the specimens were removed from the growth medium, fixed with 2.5% glutaraldehyde in 390 mosM phosphate buffer (pH 7.4) and postfixed in 1% osmium tetroxide in Veronal buffer (pH 7.3) (Warshawsky and Moore, 1967). They were then washed in the phosphate buffer and placed in an acidic gel containing 0.1 N HCl-15% gelatin (BBL) for 3.5 hours to demineralize slightly the enamel surface (Tinanoff et al., 1976). After dehydration in acetone and embedment in epoxy medium (Spurr, 1969), the resin was polymerized at 70° C.

Specimens were sectioned, re-embedded and ground as previously detailed (Tinanoff et al., 1978) so that only 1 - 5 μm of the outer surface enamel remained. Thin sections were prepared with a Reichert ultramicrotome, using a diamond knife. Silver-gold colored sections were either stained with aqueous uranyl acetate followed by lead citrate (Venable and Coggeshall, 1965) or examined unstained at 90 kV with a Zeiss EM10 electron microscope.

Transmission Electron Microprobe

Energy-dispersive x-ray analyses were performed in a JEOL JEM-100 CX transmission electron microscope equipped with a high resolution electron microscope accessory (ASID) and a Kevex Si (Li) x-ray detector connected to a Micro-X Analytical x-ray Spectrophotometer, Model 7000. The spectrophotometer was linked to a Texas Instrument Data Terminal Printer.

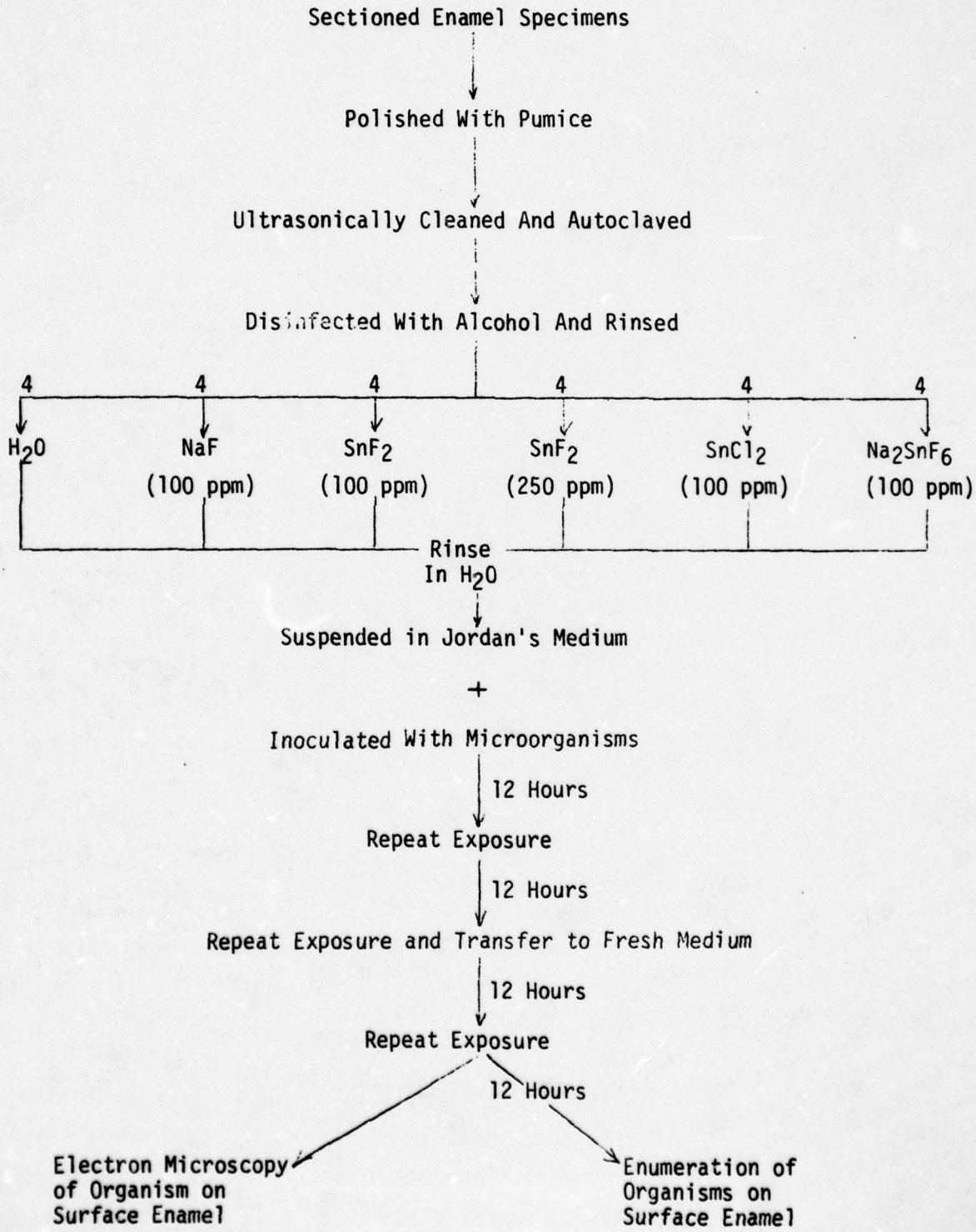
The same enamel specimens exposed intermittently to SnF_2 (250 ppm F^-) while cultured with S. mutans, were prepared for the microprobe by thin sectioning (approximately 90 nm) followed by mounting on 200 mesh formvar coated copper grids, specifically avoiding contrasting stains. Areas for analyses were located and photographed in the transmission mode (TEM), and then in the scanning mode (STEM) which was operated at an accelerating voltage of 80 KeV. From the Polaroid photographs of the STEM image, the location of the probe spot was recorded. For microprobe analysis, the specimens were tilted by means of an eucentric goniometer to 30^o for optimum collection of x-rays. The electron beam was focused to a small spot (60-100A^o) and positioned on the areas to be analyzed. X-rays emanating from these areas were counted for 100 seconds. The x-ray energy spectrum was displayed on the spectrophotometer and also recorded on Polaroid film.

Atomic Absorption Spectrophotometry

Twenty-one test tubes with rubber stoppers suspending .030" stainless steel wires (7 per treatment group) were exposed initially 1 min. either to SnF_2 (250 ppm F^-), $SnCl_2$ (Sn^{++} = Sn in 250 ppm SnF^-), or H_2O control and then washed 1 min. in water. The wires were then placed in 10 ml of Jordan's medium supplemented with 5% sucrose and inoculated with 0.1 ml of an adapted S. mutans NCTC 10449 culture. The wires were exposed as before, twice a day for 1 min., to their respective treatments with a transfer after 24 hours to fresh medium. After 48 hours (12 hours after the last exposure to the test agents), the thickness of the plaque was visually scored on each wire by the method of McCabe, Keyes and Howell, 1967, and the pH of the media from each tube was measured. The plaque from the 7 wires of each group was then pooled into one pre-weighed centrifuge tube, pelleted by centrifugation, and excess water removed. After the samples were dried, 3 days at 70^oC, the tubes were re-weighed.

For atomic absorption preparation, the plaque samples were suspended in known quantities of 10% HCl. Tin standards ($SnCl_4$, Alfa Division, Danvers, MA) were prepared at 0.1, 0.5, 1.0, 10.0, 25.0, 35.0 and 50 ppm by dilution with 10% HCl. Tin in the samples and in the standards were measured in triplicate using an atomic absorption spectrophotometer (Perkin-Elmer, Model 403) equipped with a graphite furnace (HGA-74). A deuterium discharge lamp was used to correct for non-atomic absorption signals.

Figure I. Experimental Design



RESULTS

Microbiology

The results of intermittent exposure of the various agents showed that only the 100 and 250 ppm SnF_2 significantly ($p<.05$) reduced S. mutans colonization of the enamel cylinders with a 72 and 99% reduction, respectively, compared to the water treatment controls (Table 1). Under the same experimental conditions, SnF_2 also decreased S. sanguis by 48 and 97% with 100 and 250 ppm SnF_2 (Table 2). The number of adherent A. viscosus cells was affected by all treatments; however, only SnF_2 at 100 and 250 ppm produced a significant difference from the water control (Table 3).

Among the treatment groups in each experiment there was generally a high correlation between plate counts and optical density reading ($r=0.77$). Plate counts represent only the number of viable organisms attached to the enamel; whereas, the spectrophotometric readings represent viable and non-viable organisms plus extracellular material. Close correlations among groups suggest no significant change in the amount of extracellular material or non-viable organisms in the plaque due to different treatments.

Transmission Electron Microscopy

The enamel specimens used as a control, i.e., enamel incubated for 2 days in tubes inoculated with S. mutans NCTC 10449, and exposed every 12 hours to a 1 minute water rinse, showed visible plaque deposits following osmification. The specimens intermittently treated with all the fluoride compounds at 100 ppm F^- and $SnCl_2$ also showed visible plaque formation on the enamel. However, bacterial deposits on the specimens treated with SnF_2 at 250 ppm F^- were scant

and only visible with the aid of a dissecting microscope.

Electron microscopy of the enamel specimens exposed intermittently to H_2O, NaF, SnF_2, Na_2SnF_6 (100 ppm F^-), or $SnCl_2$ revealed similar findings. Low magnification of these specimens showed morphologically normal gram positive bacteria in close apposition to the enamel. High magnification of the enamel-plaque interface characteristically showed either the bacteria slightly separated from the enamel with extracellular material associating these two structures, or the bacteria located directly on the enamel with the extracellular material often observed interposed between the bacteria and the enamel (Figures 1, 2, 3, 5, 6).

On those specimens exposed to 250 ppm F^- as SnF_2, several ultrastructural changes of the bacteria and the relationship of the bacteria to the enamel could be noted. The bacterial cell walls appeared less distinct than in the other treatment groups and there were electron dense granules and electron lucent holes seen in many of the bacterial cells. While many of the electron dense granules appeared within the cells, some electron dense material appeared to be associated with the bacterial cell wall. These electron dense granules were also noted in the specimens treated with 100 ppm SnF_2 but not as frequently. The relationship of the bacteria to the enamel also appeared altered due to a separation between the enamel and bacteria. At higher magnification, extracellular material, which characteristically mediated attachment of bacteria to enamel in the other specimens, was not as evident.

Electron Microprobe

Unstained sections of adherent S. mutans exposed intermittently to SnF_2 (250 ppm F^-) clearly showed electron dense granules in the bacteria (Figure 7). X-ray microanalyses of these granules consistently revealed peaks corresponding to the L_β peak for tin (3.67 KeV) (Figure 8). An emission peak at 3.44 KeV

coinciding with the L_α tin peak confirmed the presence of tin in these granules. Microanalysis on the bacterial cells consistently showed calcium (K_α 3.69, 3.88, K_β 4.01) in the bacterial cells (Figures 8, 9); yet, calcium was undetected in the extracellular matrix (Figure 10).

Atomic Absorption Spectrophotometry

The plaque samples prepared for tin analysis confirmed reduced bacterial colonization and metabolism in the presence of SnF_2 intermittent exposures (Table 4). Plaque accumulation on the 7 wires exposed to SnF was barely visible after 2 days; whereas, large accumulations were apparent on both the wires treated with $SnCl_2$ and on the water control wires. The character of the plaque accumulation in the $SnCl_2$ group, however, differed from the controls in regard to variability of thickness. Acid production was also reduced in those specimens exposed to SnF_2. The pH of the media at the end of the experiment in the SnF_2 group was not altered from the initial pH. Pooling the six samples from each group and subsequently obtaining the dry weight showed a large reduction of adherent plaque in the SnF_2 treated samples. This pooled sample, though, contained relatively large quantities of tin. The weight of tin per mg plaque was 11 times greater in the SnF_2 group than that found in the $SnCl_2$ group (Table 4).

able 1: Effect of exposing enamel cylinders contained in broth tubes, inoculated with S. mutans NCTC 10449, to various fluorides and controls for one minute twice a day for two days. Plate counts used to determine the number of bacteria attached to enamel after two days; optical density (spectrophotometer) used to determine amount of bacterial material attached to enamel after two days.

reatment	# of cylinders in each group	plate count (10^7) $\bar{x} \pm$ S.D.	% differences from HOH	stat. sig.	spect. readings $\bar{x} \pm$ S.D.	% differences from HOH	stat. sig.	r between plate counts and spect. readings
OH (0.0 ppmF$^-$)	4	95.6±31.0	--	*	.366±.107	--	i	.99
aF (100 ppmF$^-$)	4	81.6±12.9	-11.5	*	.358±.058	- 2.2	i	.79
nF_2 (100 ppmF$^-$)	4	26.8±14.4	-71.9	**	.172±.087	-53.1	ii	.97
nF_2 (250 ppmF$^-$)	4	1.4± 0.8	-98.5	***	.046±.009	-87.3	iii	.88
SCl_2 (100 ppm Sn^{++})	4	129.5±71.5	+36.3	*	.450±.135	+22.8	i	.56
Na_2SnF_6 (100 ppm F$^-$)	4	134.8±48.3	+41.0	*	.434±.081	+18.6	i	.24

*, **, *** Homogenous subsets using Analysis of Variance (log transformation) with Duncan procedure ($P \leq .05$)

i, ii, iii Homogenous subsets using Analysis of Variance with Duncan procedure ($P \leq .05$)

Table 2: Effect of exposing enamel cylinders contained in broth tubes, inoculated with S. sanguis ATCC 10558, to various fluorides and controls for one minute twice a day for two days. Plate counts used to determine the number of bacteria attached to enamel after two days; optical density (spectrophotometry) used to determine amount of bacterial material attached to enamel after two days.

treatment	# of cylinders in each group	plate count (10^6) $\bar{X} \pm$ S.D.	% differences from HOH	stat. sig.	spect. readings $\bar{X} \pm$ S.D.	% differences from HOH	stat. sig.	r between plate counts and spect. readings
HOH (0.0 ppmF^-)	4	138.0±84.7	--	*	.222±.055	--	i	.86
NaF (100 ppmF^-)	4	158.1±26.0	+14.6	*	.199±.048	-10.4	i,ii	.26
SnF_2 (100 ppmF^-)	4	72.2±64.3	-47.7	**	.141±.094	-36.5	i,ii	.97
SnF_2 (250 ppmF^-)	4	4.4± 7.8	-96.8	**	.052±.020	-76.6	iii	.94
$SnCl_2$ (100 ppmSn^{++})	4	99.5±38.0	-27.9	*	.150±.013	-32.4	i,ii	.61
Na_2SnF_6 (100 ppmF^-)	4	103.6±44.3	-24.9	*	.114±.040	-48.6	ii,iii	.74

*, ** Homogenous subsets using Analysis of Variance (log transformation) with Duncan procedure ($P \leq .05$)

i,ii,iii Homogenous subsets using Analysis of Variance with Duncan procedure ($P \leq .05$)

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Table 3: Effect exposing enamel cylinders, contained in broth tubes inoculated with A. viscosus M 100, to various fluorides and controls for one minute twice a day for two days. Plate counts used to determine the number of bacteria attached to enamel after two days; optical density (spectrophotometry) used to determine amount of bacterial material attached to enamel after two days.

treatment	# of cylinders in each group	plate count (10^6) $\bar{x} \pm$ S.D.	% differences from HOH	stat. sig.	spect. readings $\bar{x} \pm$ S.D.	% differences from HOH	stat. sig.	r between plate counts and spect. readings
HOH (0.0 ppmF^-)	4	302.8±166.7	--	*	.276±.07	--	i	.98
NaF (100 ppmF^-)	4	123.3± 57.1	-59.3	*	.208±.09	-25.4	i	.99
SnF_2 (100 ppmF^-)	4	28.7± 31.0	-90.5	**	.079±.04	-71.4	ii,iii	.98
SnF_2 (250 ppmF^-)	4	1.0± 1.8[a]	-99.7	***	.020±.06	-92.8	ii	.24
$SnCl_2$ (100 ppmSn^{++})	4	195.3± 67.9	-35.5	*	.209±.08	-24.3	i	.91
Na_2SnF_6 (100 ppmF^-)	4	152.5± 80.2	-49.6	*	.190±.113[b]	-31.2	i,iii	.99

a 2 specimens in which no C.F.U.'s were detected on 10^5 plates were recorded as 1 x 10^5

b 3 instead of 4 specimens due to laboratory accident

*,**,*** Homogenous subsets using Analysis of Variance (log transformation) with Duncan procedure ($P \leq .05$)

i,ii,iii Homogenous subsets using Analysis of Variance with Duncan procedure ($P \leq .05$)

Table 4: S. mutans NCTC 10449 growth on wires exposed every 12 hr. for 1 min. to various agents. After 2 days the plaque thickness on 7 wires in each treatment group was scored, then pooled, dried, and analyzed for tin.

	Terminal pH of broth	Plaque score $^{\alpha}$	Dry Plaque Weight (mg)	Sn++/ Total Sample (ppm)	Sn++/ mg plaque (μg)
H_2O	4.6	4	88.4	ND$^{\beta}$	ND
$SnCl_2$ (Sn++ equimolar to SnF_2)	4.7	4$^{\gamma}$	32.7	58	1.8
SnF_2 (250 ppm F^-)	7.7	<1	0.6	12	20.0

α Scored by McCabe method

β None detected

γ Variable in thickness

Discussion

The present series of experiments, designed to further examine the antiplaque determinants of SnF_2, have shown that SnF_2 reduces the ability of several oral plaque-forming microorganisms to colonize enamel. The magnitude of antiplaque properties was related to concentrations of SnF_2 used; however, the effect cannot be ascribed to bactericidal properties since high numbers of viable microorganisms were still recoverable from the enamel of those specimens treated with 250 ppm SnF_2. Furthermore, the acidity of SnF_2, per se, appears not to be the critical antiplaque factor. Agents with lower pH values than SnF_2, i.e., $SnCl_2$ showed no significant effect in reducing bacterial colonization of the enamel cylinders.

Previous studies have suggested that individual ion species of SnF_2 are not by themselves as effective as when these ions are in combination. Neither stannous ions as $SnCl_2$, nor fluoride ions as NaF appeared as potent as SnF_2 in decreasing the amount of plaque (Tinanoff, Brady, Gross, 1976). The present results which also show more substantial antiplaque properties for SnF_2 provide additional support for the previous findings. The reason for the increased effectiveness of SnF_2 may be explained by results of the electron microprobe and chemical analyses in the present study. The electron dense granules in those bacteria treated with SnF_2, identified by electron microprobe as tin deposits, suggest that plaque exposed to SnF_2 accumulates tin. These morphologically characteristic tin granules have also been retrospectively noted in electron micrographs of plaque from human subjects rinsing twice daily with 100 ppm SnF_2 (Tinanoff et al., 1976). The chemical analyses showing high levels of tin

in plaques exposed to SnF_2 further confirms tin retention when this element is associated with fluoride.

The difference in bacterial cell uptake of fluoride and chloride ions may partially explain the observations of tin deposition in plaque treated with SnF_2 but not $SnCl_2$. Many authors have reported higher concentrations of fluoride in dental plaque than in the surrounding oral fluids (Hardwick and Leach, 1962; Dawes, Jenkins, Hardwick and Leach, 1965; Jenkins and Edgar, 1969). This fluoride accumulates only within the bacterial cells (Jenkins and Edgar, 1969). However, chloride apparently is not concentrated in bacteria (Schultz, Wilson, Epstein, 1962; Mitchell and Moyle, 1959). Hence, the large accumulation of tin found in the bacterial cells exposed to SnF_2 could possibly be explained by the transport and accumulation of fluoride in the cells, with tin passively entering the cells coupled to the fluoride ions. The hypothesis of undissociated SnF_2 transport across the cell membrane does not coincide with those of Whitford et al. (1977) who suggested that fluoride enters the cell only as HF.

Further information on the mechanisms by which SnF_2 effects a reduction in the amount of plaque may be derived from the electron microscopic findings in the present study which suggest both an alteration in bacterial growth as well as an alteration of bacterial attachment from SnF_2. In addition to the observation that bacteria exposed to 250 ppm SnF_2 have indistinct cell walls, these bacteria frequently were noted to have intracellular electron lucent holes. Such holes are compatible with the artifact which is seen when bacterial polyphosphate is examined under the electron microscope (Voelz, Voelz, and Ortigoza, 1966). Bacterial polyphosphate is believed to be formed in cells when conditions are not favorable to growth (Harold, 1966). The large number of intracellular holes found in the SnF_2 treated specimens may indicate unbalanced growth. Heavy metals, such as tin, are known to be "germicides" by virtue of their ability to precipitate protein (Salle, 1968). Thus, the tin found in

the cells may well be metabolically disruptive and produce the noted altered cell appearances.

Ultrastructurally, plaque attachment in those specimens treated with 250 ppm SnF_2 also appeared different from that found in the other treatment groups. The bacteria in the 250 ppm SnF_2 treated group appeared separated from the enamel surface and extracellular material associated with bacterial attachment (Tinanoff et al., 1978) was not apparent. These findings are consistent with electron microscopic observation of human dental plaque treated with twice daily SnF_2 mouthrinses which also showed bacterial separation from the enamel (Tinanoff et al., 1976).

The tin ions also may be effective in altering bacterial attachment by competing with calcium for acidic groups on the bacterial surface or acidic groups on the pellicle or on the tooth itself (Svatun et al., 1977; Rölla, 1976). The observation in this study of tin accumulation on the bacterial cell walls, as well as intracellularly, is compatible with this concept. Additionally, others have noted that SnF_2 reduces the surface tension at the air-water interface of enamel (Glantz, 1969). These adhesion reducing properties of SnF_2 may contribute to the diminished plaque formation.

An important feature of presently known antiplaque agents is their ability to bind to oral structures, which aids in oral retention. The presence of tin on and within plaque bacteria exposed only briefly to dilute solutions of SnF_2 suggests that tin accumulates and is retained in plaque. It is noteworthy that the tin is apparently concentrated into granules in the bacteria. This granule formation is not unlike other cell responses to heavy metals which is believed a method by which cells "detoxify" foreign elements (Simkiss, 1977). The tin accumulation may be the most important antiplaque determinant of SnF_2.

Plate 1

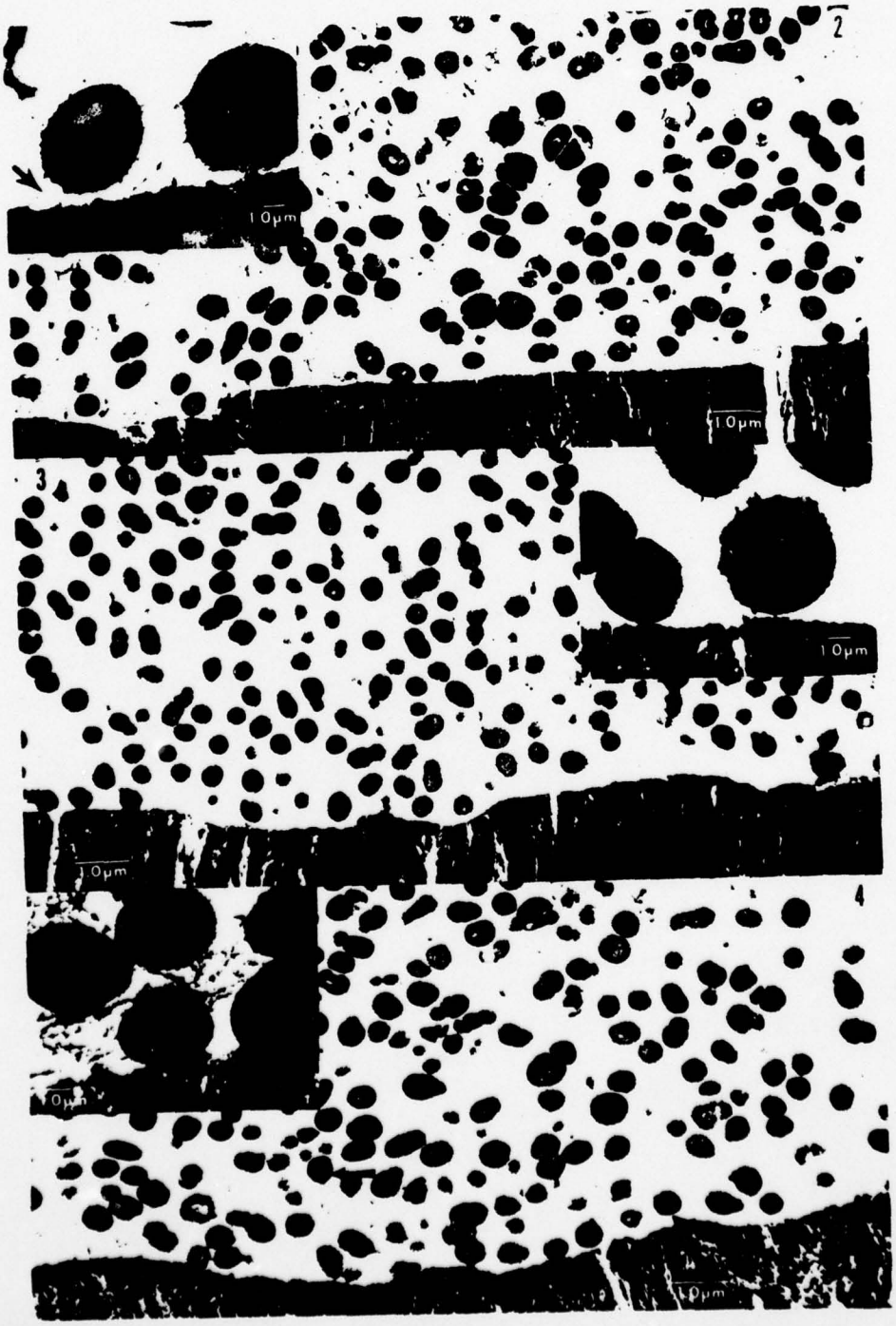

Plate 1

Figure 2. Electron microscopy of S. mutans, NCTC 10449, attachment to enamel in vitro. Here suspended human enamel, incubated in inoculated complex medium supplemented with 5% sucrose, was exposed to a 1 minute water rinse (control) at 12 hour intervals. After 48 hours, the enamel with attached bacteria was fixed and processed for electron microscopy. Low magnification shows morphologically normal bacteria in close apposition to the enamel. X 7,800; uranyl acetate, lead citrate stains. Inset is higher magnification showing extracellular material (arrow) mediating attachment of the bacteria to enamel. X 40,000; uranyl acetate, lead citrate stains.

Figure 3. Electron microscopy of enamel suspended in media inoculated with S. mutans, exposed to NaF (100 ppm F^-) for 1 minute at 12 hour intervals, and then fixed after 48 hours. Low magnification shows bacteria close to enamel similar to that noted for the water control. X 9,100; uranyl acetate, lead citrate stains. High magnification inset shows bacteria attached to enamel. X 40,000; uranyl acetate, lead citrate stains.

Figure 4. Electron microscopy enamel suspended in media, inoculated with S. mutans, exposed to SnF_2 (100 ppm F^-) for 1 minute at 12 hour intervals, and then fixed after 48 hours. Low magnification shows bacteria close to enamel similar to that noted for the water control. An electron dense granule is noted in one bacterium (arrow) X 9,100; uranyl acetate, lead citrate stains. Inset shows bacterial attachment to enamel mediated by an extracellular material. X 40,000; uranyl acetate, lead citrate stains.

Plate 2

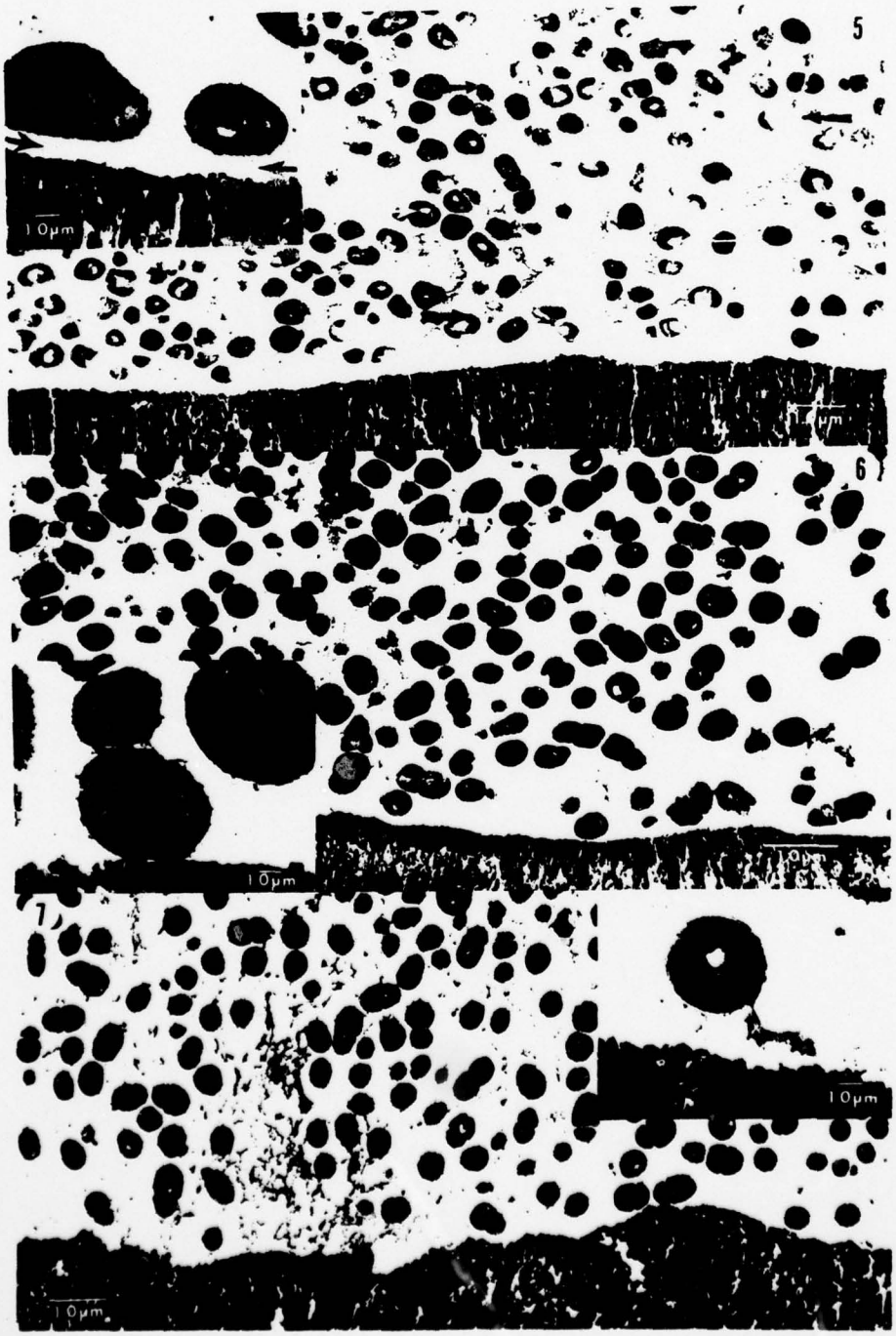

Plate 2

Figure 5. Electron microscopy of enamel suspended in media inoculated with S. mutans, exposed to SnF_2 (250 ppm F^-) for 1 minute at 12 hour intervals, and then fixed after 48 hours. Low magnification besides showing as apparent separation of bacteria from the enamel, reveals that there are numerous electron dense granules on and within the bacterial cells (black arrows). Artifactual holes compatible with polyphosphate material are also present in the cells (white arrows) X 9,100; uranyl acetate, lead citrate stains. High magnification shows 2 bacteria with indistinct cell walls, one also has an electronlucent enamel, nor joined to it by extracellular material (arrows). X 40,000; uranyl acetate, lead citrate stains.

Figure 6. Electron microscopy of enamel suspended in media inoculated with S. mutans, exposed to $SnCl_2$ (Sn++ = Sn++ in 100 ppm SnF^-) for 1 min. at 12 hour intervals, and then fixed after 48 hours. Low magnification shows bacteria close to enamel similar to that noted for the water control. X 12,000; uranyl acetate, lead citrate stains. High magnification inset shows bacteria attached to enamel. X 40,000; uranyl acetate, lead citrate stains.

Figure 7. Electron microscopy of enamel suspended in media inoculated with S. mutans, exposed to NaF (100 ppm F^-) for 1 minute at 12 hour intervals, and then fixed after 48 hours. Low magnification shows bacteria close to enamel similar to that noted for water control. X 9,100; uranyl acetate, lead citrate stains. High magnification inset distinctly shows extracellular material mediating attachment of the bacteria to enamel. X 40,000; uranyl acetate, lead citrate stains.

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THE EFFECT OF A LOW FLUORIDE DELIVERY SYSTEM ON BACTERIAL METAB--ETC(U)
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Plate 3

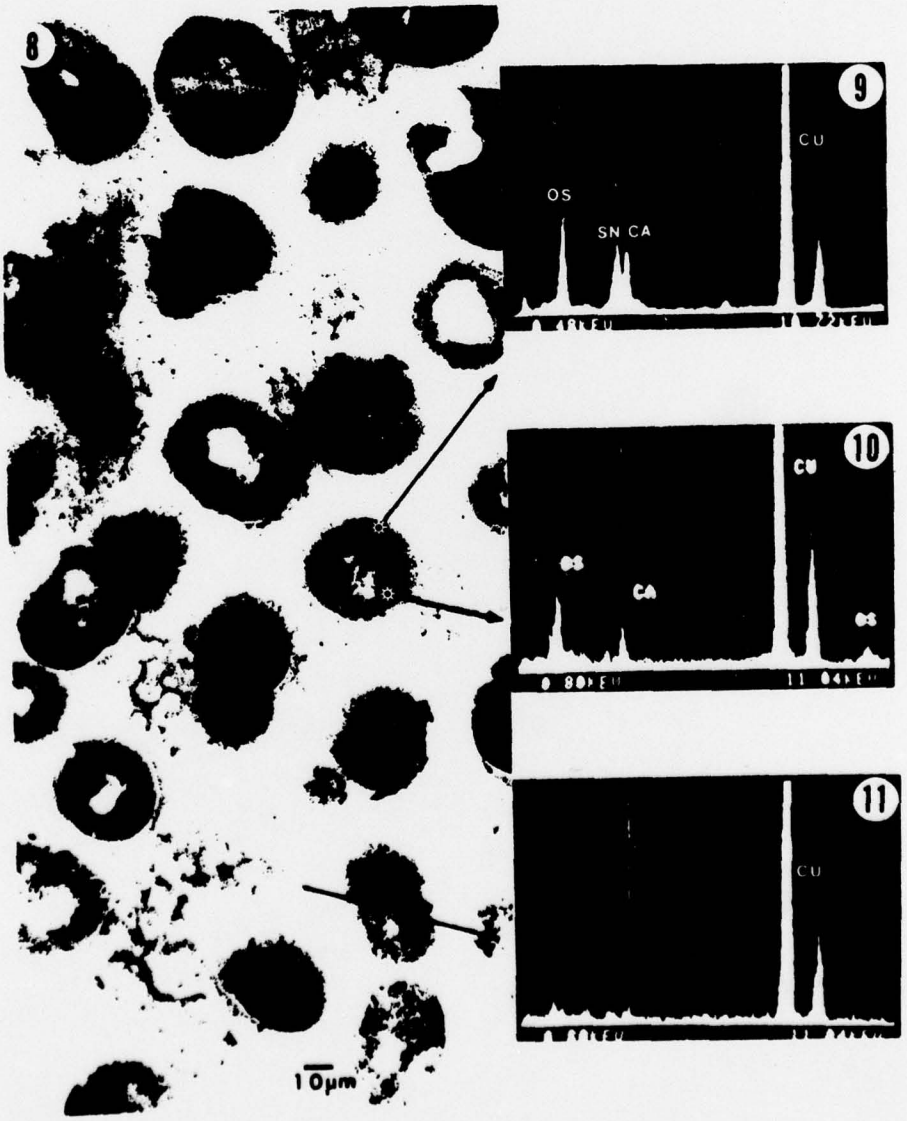

Plate 3

Figure 8. Electron microscopy of the bacteria exposed to SnF_2 (250 ppm F^-) for 1 minute at 12 hour intervals, and then fixed after 48 hours. Specimen is the same as that of Figure 4 except that no contrasting stains were used in order to prevent extraneous interference with energy-dispersive X-ray microanalyses. Note prevalence of electron dense granules and electron lucent holes as also shown in Figure 4. X 40,000; no stains.

Figure 9. X-ray energy spectrum of electron dense granule noted within the bacterial cells. Note tin (SN), with an L_α peak of 3.44 KeV, and calcium (CA), K_α 3.69 KeV, are evident along with background of osmium (M_α 1.91, M_β 1.97) and copper (K_α 8.027, 8.047; K_β 8.904, 8.976).

Figure 10. X-ray energy spectrum within the bacterial cell, but not on the electron dense granules. Only the calcium peak along with backgrounds of osmium and copper are present. Vertical white lines indicate position of the K_α and K_β lines of calcium.

Figure 11. X-ray energy spectrum of the extracellular matrix. Neither calcium nor tin is detected in this area.

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