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Fluoride Remineralization of Demineralized Bovine Tooth Enamel and Hydroxyapatite Pellets

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Received June 30, 1980, from the **College of Pharmacy, University of Michigan, Ann Arbor, MI 48109*, and the ‡*American Dental Association, Chicago, IL 60611.* Accepted for publication January 26, 1981.

Abstract □ Both bovine enamel and hydroxyapatite pellets were remineralized in a fluoride-containing remineralization solution after prior demineralization for various lengths of time. In both the enamel and pellet systems, the degree of remineralization attainable was directly related to the extent of prior demineralization, although the demineralized material was never 100% recovered in remineralization. In some cases, fluoride levels up to several thousand parts per million were found at depths as great as 50 μm from the surface. The stoichiometry of the remineralized material and electron microprobe examination were consistent with the formation of fluoridated hydroxyapatite rather than calcium fluoride.

Keyphrases □ Fluoride—remineralization of demineralized bovine tooth enamel and hydroxyapatite pellets □ Remineralization—of demineralized bovine tooth enamel and hydroxyapatite pellets □ Hydroxyapatite pellets—remineralization along with demineralized bovine tooth enamel □ Teeth, bovine—remineralization of demineralized tooth enamel and hydroxyapatite pellets

Previous studies performed in these laboratories (1, 2) led to the development of a model for hydroxyapatite dissolution involving two distinct crystalline dissolution sites (3). This model is consistent with dissolution kinetic data and explains the observed morphology of hydroxyapatite dissolution at both the single-crystal level (4) and at the level of a compressed hydroxyapatite pellet or a block of tooth enamel (3).

In studying demineralization of remineralized enamel, it was noted (5, 6) that when enamel was demineralized under conditions where the medium was partially saturated with respect to hydroxyapatite, subsequent remineralization was successful; but when prior demineralization was carried out under more severe conditions, remineralization attempts gave poor results.

Since successful remineralization occurred under conditions where dissolution *via* only Site 1 would be expected, the hypothesis follows that this site might be the principal one for successful remineralization. This remineralization might consist chiefly of the filling of *c*-axis holes.

The present experiments were designed to test for the existence of a relationship between the amount of demineralization *via* Site 1 and the subsequent degree of remineralization attainable.

EXPERIMENTAL

Materials—*Bovine Teeth*—Bovine incisors were used. Labial surfaces were ground with fine sandpaper to remove the pellicle.

Hydroxyapatite Pellets—Synthetic hydroxyapatite crystals, prepared using the procedure developed by Moreno *et al.* (7), were used in the preparation of pellets. About 50 mg of hydroxyapatite, preequilibrated in a humidity chamber containing saturated potassium nitrate aqueous solution to maintain the humidity at ~67%, was compressed in a die (0.62-cm diameter) under a force of 10,000 lb using a laboratory press.

Buffer Solutions—A solution 16% saturated (on a molar basis) with respect to the thermodynamic solubility of hydroxyapatite was used for demineralization. The solution was a 0.1 *M* acetate buffer containing 3.5 m*M* each of total calcium and total phosphate. The pH was adjusted to 4.5 with sodium hydroxide, and the ionic strength was adjusted to 0.5 with sodium chloride. For remineralization, a 0.1 *M* acetate buffer (pH 4.5) containing 12 m*M* of total calcium (including calcium 45 activity of 0.2 mCi/liter of solution), 12 m*M* total phosphate, 10 ppm of fluoride, and sodium chloride to adjust the ionic strength to 0.5 was used.

The ionic activity of hydroxyapatite, K_{HAP} ($\equiv a_{Ca}^{10} a_{PO_4}^{6} a_{OH}^{2}$), of this buffer was 10^{-120}. Although not saturated with respect to hydroxyapatite, this solution has a high enough degree of partial saturation so that hydroxyapatite dissolution would be expected to proceed slowly, if at all, based on previous work with solutions approaching this level of calcium and phosphate concentrations (3, 8).

Demineralization—A bovine tooth was covered with inlay wax except for 0.25 cm^2 of the labial surface. In the pellet experiments, a hydroxyapatite pellet with a surface area of 0.58 cm^2 was fixed on a small glass plate by the wax. Either the tooth or pellet then was demineralized in the 16% partially saturated buffer solution for 0.5–6 hr. The solution was shaken gently with a wrist-action shaker[1] and kept at 30°. The volume of the buffer solution was 10 ml/0.25 cm^2 of the sample surface.

Remineralization—The demineralized tooth or pellet then was given a remineralization treatment for 24–72 hr in 50 ml of the remineralization buffer solution. The solution was shaken gently and kept at 30° as remineralization proceeded.

Etching and Scraping—The remineralized sample then was etched in 1 ml of 0.5 *M* $HClO_4$ for successive periods of 15, 15, 30, 60, 120, 200, and 200 sec; the surface was washed with 1 ml of water after each etching step. The depths of the etched layers were estimated from the amounts of phosphate etched. Some hydroxyapatite pellets were scraped evenly

[1] Burrell Co.

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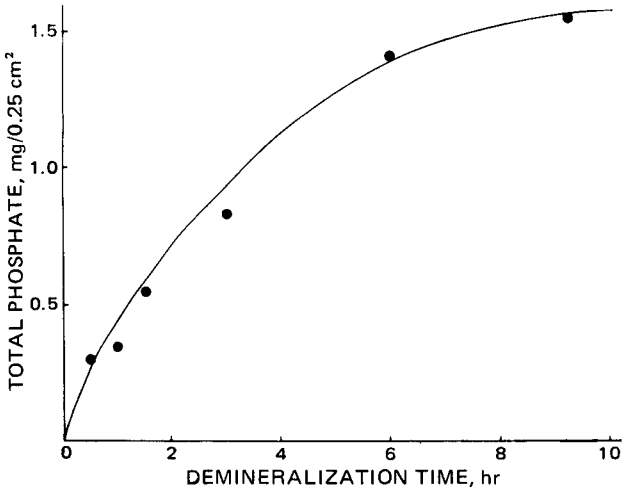

Figure 1—*Total phosphate dissolved from bovine tooth as a function of demineralization time.*

by a sharp razor in lieu of etching. The depths of the scraped layers were estimated from the weights of the powdered scrapings. This powder then was dissolved in perchloric acid and assayed to determine fluoride and calcium 45 concentrations.

Analytical Techniques for Phosphate, Fluoride, Calcium, and Calcium 45—Phosphate concentrations were determined by the method of Gee *et al.* (9), in which the phosphoammonium–molybdate complex formed is reduced by stannous chloride. The absorbance of the resulting blue color is determined after 15 min at 720 nm[2]. Fluoride concentrations were determined by a fluoride electrode[3] using a low level total ionic strength-adjusting buffer. Concentrations of calcium 45 were determined by a scintillation counter[4].

Depth of Etchings—The depths of the etched layers of the teeth and hydroxyapatite pellets were calculated from the amounts of phosphate removed in the etchings, assuming the density of the samples to be 2.95 g/ml. This information then was used to calculate the fluoride and calcium 45 content of the mineral per unit volume.

Recovery of Demineralized Mineral by Remineralization—The extent of remineralization is defined by the molar ratio of the total amount of fluoridated hydroxyapatite formed during remineralization to the amount of hydroxyapatite dissolved during demineralization. In this study, the molar ratio was defined as the cumulative calcium 45 taken up during remineralization to the calcium dissolved during demineralization.

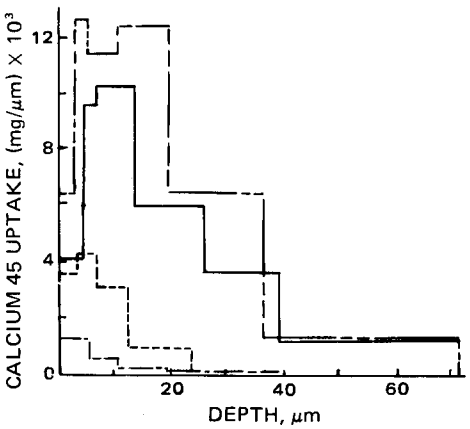

Figure 2—*Influence of demineralization time on 24-hr calcium 45 uptake profiles in bovine teeth. The D and R represent demineralization and remineralization, respectively. The lower subscripts represent the time in hours. Key: ——·——, D_6–R_{24}; —, D_3–R_{24}; ---, D_1–R_{24}; and —·—, D_0–R_{24}.*

[2] Hitachi 690 spectrophotometer.
[3] Model 94-09, Orion Co.
[4] Liquid scintillation system, Beckman Instruments.

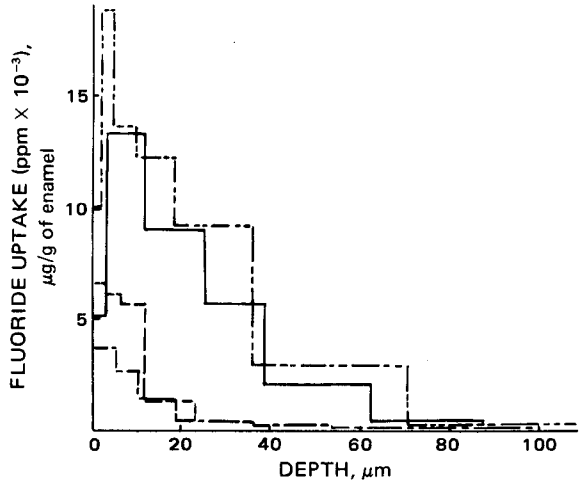

Figure 3—*Influence of demineralization time on 24-hr fluoride uptake profiles in bovine teeth. Key: ——·——, D_6–R_{24}; — , D_3–R_{24}; ---, D_1–R_{24}; and —·—, D_0–R_{24}.*

RESULTS

Effect of Demineralization on Remineralization—Bovine teeth were demineralized in 10 ml of the 16% saturated buffer solution for 0–6 hr. Figure 1 shows the total phosphate (milligrams) dissolved per 0.25 cm² of surface. These results show that the buffer solution was not yet saturated with respect to hydroxyapatite after 6 hr of demineralization. That is, dissolution was still occurring.

To investigate the effects of demineralization on subsequent remineralization, the bovine teeth demineralized for 0–6 hr were remineralized for 24 hr. Figure 2 shows calcium 45 concentration profiles for these teeth. The calcium 45 concentration increases with increasing demineralization times, and the enamel not subjected to prior demineralization (D_0) was remineralized to a negligible extent. Figure 3 shows the fluoride concentration profiles of the same samples. Fluoride concentrations also increased with increasing demineralization times. However, the fluoride uptake of nondemineralized enamel was not negligible as was the calcium 45 uptake, probably as a result of a higher tendency for superficial ion exchange.

The fluoride concentration gradient of the teeth demineralized less than 1 hr decreased rapidly from the high levels at the surface to <500 ppm at a depth of only 20 μm, but the teeth demineralized more than 1.5 hr had much higher fluoride gradients, decreasing gradually to ~1000–2000 ppm of fluoride at depths of ~50–60 μm. These results are consistent with results in which teeth demineralized under mild conditions are rehardened or remineralized much easier than sound teeth (10, 11).

The effect of remineralization time on the calcium 45 and fluoride uptake was examined by remineralizing the teeth for 24 and 72 hr following 6 hr of demineralization. Figure 4 shows the resulting calcium 45 concentration profiles. They were nearly the same with remineralization being essentially complete at 24 hr.

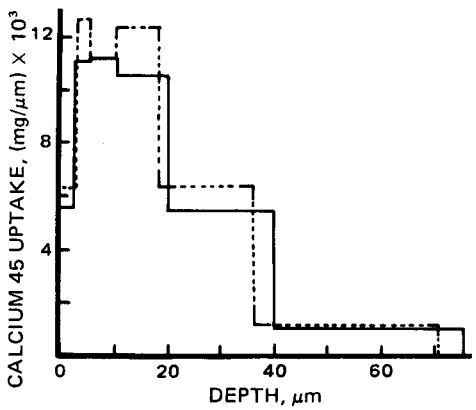

Figure 4—*Influence of remineralization time of calcium 45 uptake profiles in bovine teeth, showing that remineralization is essentially complete in 24 hr. Key: ---, D_6–R_{24}; and —, D_6–R_{72}.*

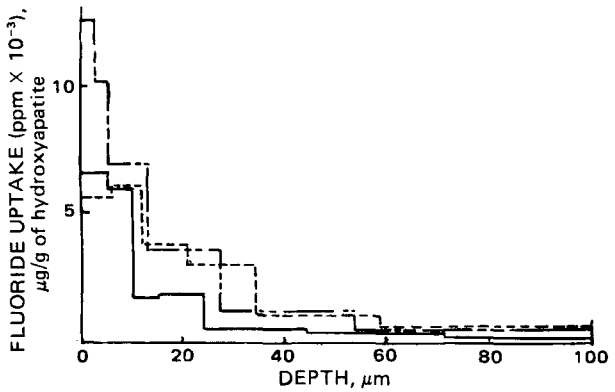

Figure 5—*Influence of demineralization time on 24-hr fluoride uptake profiles in hydroxyapatite pellets as measured by the perchloric acid etch technique. Key: —·—, $D_{3.0}$–R_{24}; ---, $D_{1.5}$–R_{24}; and —, $D_{0.0}$–R_{24}.*

To confirm that the demineralization/remineralization dependence seen in bovine teeth was indeed a result of hydroxyapatite behavior as expected from the model and not caused primarily by the enamel matrix, the remineralization of hydroxyapatite pellets was examined by the same method as used for the bovine teeth. Hydroxyapatite pellets were demineralized for 0, 1.5, or 3 hr. Figure 5 shows the fluoride concentration profiles obtained after remineralizing these pellets for 24 hr. The concentrations increased with increasing demineralization time in accord with the results obtained with bovine teeth. Although the calcium 45 uptake data are now shown, they were also coincident with the enamel data. However, fluoride and calcium 45 concentrations in the nondemineralized pellet were noticeably higher than those observed in enamel.

Mineral Recovery by Remineralization—The extent of mineral recovery was calculated in several experiments with bovine enamel and hydroxyapatite pellets (Fig. 6). In no case was the demineralized mineral completely replaced during remineralization.

Form of Incorporated Fluoride—If fluoridated hydroxyapatite was formed from the ions supplied by the remineralization solution, the molar ratio of calcium 45 to fluoride uptake should be 5.0; a ratio of 0.5 would be expected for the formation of calcium fluoride (I). Figure 7 shows the molar ratio of calcium 45 to fluoride uptake after 24 hr of remineralization for both bovine teeth and hydroxyapatite pellets as a function of demineralization time. The ratios were ~5.0, corresponding to the formation of fluoridated hydroxyapatite. However, for demineralization of <1.5 hr, the ratios were <5 but much higher than the 0.5 ratio expected from the formation of I. This discrepancy from 5.0 might be due to the formation of I to a small extent or to the ion exchanges of calcium and hydroxide ions of hydroxyapatite for calcium 45 and fluoride in the buffer solution.

In a remineralization study with hydroxyapatite powder using a remineralizing solution identical to that described earlier, it was shown by extraction with potassium hydroxide that the fluoride in the remineralized material is not in the form of I and is most likely apatitic (12).

As an independent check of the results obtained by the etching method,

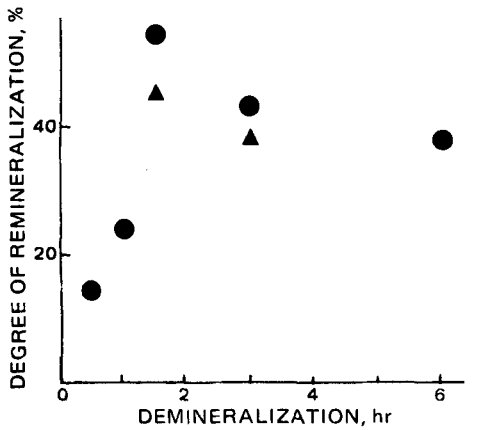

Figure 6—*Influence of demineralization time on the degree of subsequent remineralization. Key: ●, bovine tooth; and ▲, hydroxyapatite pellet.*

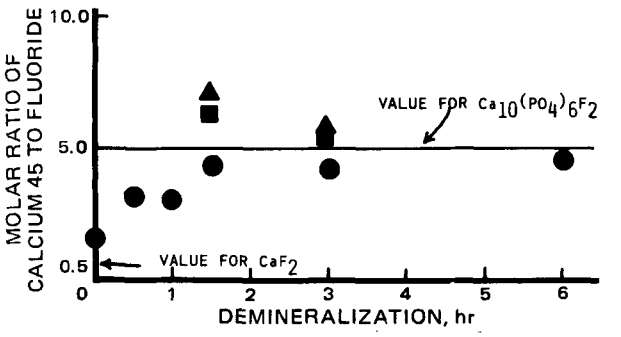

Figure 7—*Influence of demineralization time on the calcium 45 to fluoride molar ratio of the subsequently remineralized phase. Key: ●, bovine tooth (etching method); ■, hydroxyapatite pellet (etching method); and ▲, hydroxyapatite pellet (scraping method).*

the remineralization of hydroxyapatite pellets was investigated by the scraping method. Pellets remineralized for 24 hr following 1.5 or 3.0 hr of demineralization were scraped to a depth of ~60 μm, and the scrapings were analyzed for fluoride and calcium 45. The uptakes of fluoride and calcium 45 measured in this way were almost identical to those obtained by the etching method. The molar ratios of calcium 45 to fluoride uptake obtained from the etching and scraping techniques are shown in Fig. 7. The coincidence of the results of the two methods confirms the results obtained from the etching method. As a further check, electron microprobe determinations (calcium, phosphate, and fluoride) as a function of depth were carried out on sectioned, remineralized bovine teeth. The results were in good agreement with the perchloric acid method.

All experiments were carried out in duplicate, with the variation between the two trials typically on the order of 10–15%.

DISCUSSION

In both bovine enamel and compressed hydroxyapatite pellets, there was a direct relationship between the amount of mineral lost during demineralization and the amount regained during remineralization for the time periods studied. In all cases, there was greater uptake of calcium 45 and fluoride in the pellet than in the bovine enamel. This difference is likely due to the higher porosity of the pellet. These results are consistent with the idea that when there is no prior demineralization, remineralization is restricted to available void spaces in the pellet or enamel. During demineralization, however, additional spaces are created (including *c*-axis holes in the crystals) in which remineralization can occur. It appears that remineralization is occurring both in the initial voids and in the voids created by demineralization.

The extent of remineralization after a 24-hr remineralization treatment was considerably less than 100% both for bovine teeth and hydroxyapatite pellets. However, in the real *in vivo* situation where teeth are continuously undergoing demineralization/remineralization cycles, the extent of remineralization must, on the average, be 100% unless there is an ongoing net growth of lesions.

It was expected that for longer demineralization times, the recovery during remineralization might be <100% because many more crystals would have time to dissolve completely, leaving no templates for subsequent remineralization. Work currently in progress in these laboratories, using microradiography as a tool for measuring mineral density profiles (13), has shown that this is the case. The described demineralization conditions result in a recession of the surface due to complete removal of ~40 μm of enamel, below which there is a lesion ~40 μm deep that is completely filled during the subsequent remineralization treatment.

This mechanism implies that for short demineralization times, nearly 100% recovery might be expected. In actuality, for the shortest demineralization times (<1.5 hr), the extent of remineralization was less than for some longer demineralization times. One possible explanation is that the starting enamel or pellet was not uniform and that the very first material removed in the demineralization was some more soluble phase that did not dissolve *via* the hole-forming mechanism presumably followed by hydroxyapatite under these conditions and, hence, did not leave any such templates for remineralization.

CONCLUSIONS

Both bovine enamel and hydroxyapatite pellets were remineralized in a fluoride-containing remineralization solution after prior demineral-

alization for various lengths of time. In both the enamel and pellet systems, the degree of remineralization attainable was directly related to the extent of prior demineralization, although in no instance was the demineralized material 100% recovered in remineralization. Fluoride levels up to several thousand parts per million were found at depths as great as 50 μm from the surface in some cases. The stoichiometry of the remineralized material and electron microprobe examination were consistent with the formation of fluoridated hydroxyapatite rather than I. The detailed characterization of the behavior of the remineralized material is now being addressed in studies in these laboratories.

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Novel Topical Fluoride Delivery System I: Remineralization of Ground Bovine Teeth

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Received June 30, 1980, from the **College of Pharmacy, University of Michigan, Ann Arbor, MI 48109*, and the ‡*American Dental Association, Chicago, IL 60611.* Accepted for publication January 26, 1981.

Abstract □ Laboratory studies were carried out on a newly conceived fluoride-containing remineralizing system with bovine teeth. The prototype fluoride delivery device involved micronized calcium fluoride maintained at the tooth surface with a cellulose film. Together with salivary calcium and phosphate (or simulated saliva), this system was able to generate and maintain the appropriate thermodynamic activity driving force for significant fluorapatite deposition in a reasonably short time (~48 hr).

Keyphrases □ Delivery devices—fluoride, remineralization of bovine teeth, effect of film thickness and particle size □ Fluoride—delivery devices for remineralization of bovine teeth, effect of film thickness and particle size on remineralization □ Remineralization—bovine teeth, fluoride delivery devices, effect of film thickness and particle size on remineralization □ Teeth, bovine—remineralization using fluoride delivery devices, effect of film thickness and particle size on remineralization

Recent *in vitro* studies in these laboratories (1) showed that the amount of fluoride incorporated in a remineralization treatment can be increased substantially if the tooth is demineralized carefully prior to the remineralization. This finding suggests that successful remineralization might be attained *in vivo* if the teeth could be demineralized to the same extent as in the *in vitro* experiments. This paper describes the initial studies in the development of a fluoride topical delivery system designed to achieve *in vivo* results similar to the results obtained *in vitro*.

In vitro remineralization can be very successful when the ionic activity product, K_{FAP} ($a^{10}_{Ca^{2+}} a^{6}_{PO_4^{3-}} a^{2}_{F^-}$), is $\sim 10^{-108}$ (1). Less concentrated solutions provide less driving force for remineralization; more concentrated solutions may result in the rapid precipitation of calcium fluoride (I) or dicalcium phosphate dihydrate (II) in the prepared solutions themselves or in enamel pores, thereby blocking or retarding remineralization. It was decided to control the solution conditions at the enamel surface by supplying fluoride in the form of I suspended in a film adhering to the enamel surface.

Calculations have shown that mixtures of I and II or I alone in the film should result in solution compositions appropriate for remineralization at the enamel surface. Furthermore, the relatively low solubility of I limits the rate at which the suspended particles dissolve, so that fluoride applied in this way is inherently long acting, even with no moderation from the film. This fact makes the film design problem much simpler than when a more soluble fluoride source such as sodium fluoride is used and the film must then control the release rate. Therefore, the problem of film design is not to find a film with a narrowly prescribed set of properties but rather to find a film that can hold the particles of I in place while being porous to saliva and interfering minimally with particle dissolution.

EXPERIMENTAL

Materials and Methods—Bovine teeth from 8-week-old, crate-fed, strictly kosher calves were obtained from packing houses in the Chicago area. These animals were chosen because each is subjected to rigidly controlled and uniform environmental conditions (including diet) and