

CYCLICAL ASPECTS OF ENAMEL MATURATION AND THE ROLE OF RUFFLE-ENDED AND SMOOTH-ENDED AMELOBLASTS

M.D. McKEE,¹ A. NANJI,² C.E. SMITH³ AND H. WARSHAWSKY⁴

¹Physical Chemistry Department, Forsyth Dental Center, 140 The Fenway, Boston, MA, USA 02115,

²Departments of Anatomy and Stomatology, Université de Montréal, and Departments of ³Oral Biology and

⁴Anatomy, McGill University, Montreal, Quebec, Canada.

INTRODUCTION

During the maturation stage of amelogenesis, two major changes occur within the enamel: 1) there is degradation and removal of organic matrix components previously accrued in the secretory stage, and 2) there is a massive influx of Ca and P mineral ions. Although the exact mechanisms by which these changes are achieved remain to be clarified, recent evidence suggests that precise cyclical cellular control is exerted over these maturative events by ruffle-ended and smooth-ended ameloblasts.¹⁻⁷

MATERIALS AND METHODS

Light microscopy Rats (100 g) were fixed by intracardiac perfusion with 5% glutaraldehyde in 0.08 M sodium cacodylate buffer containing 0.05% CaCl₂, pH 7.3. Lower incisors were dehydrated, embedded in Epon, sectioned, and stained with toluidine blue.

Whole-mount radioautography (RAG) Rats (100 g) were injected with 1 μ Ci/gbw of either ⁴⁵Ca (as calcium chloride or ³³P (as orthophosphoric acid) and sacrificed by decapitation after 5 min. Dissected incisors were wiped free of their enamel organs and coated with emulsion and processed for RAG as described previously.¹

In vitro Ca and protein staining Staining of Ca with glyoxal bis[2-hydroxyanil](GBHA), arsenazo III, and calmagite was performed as described previously^{2,3} on rat, rabbit and pig teeth from 1-, 3- and 6-month-old animals, respectively. Proteins in the surface layer of the enamel were visualized by staining with uranyl acetate and Coomassie blue and by whole-mount RAG 1 hr after ³⁵S-methionine injection.⁴

Vinblastine experiment Rats (100 g) were injected intravenously with 0.5 mg vinblastine and sacrificed at 4 and 8 hr, stained with GBHA, or additionally injected with ⁴⁵Ca or calcein⁵ at 4 and 8 hr after vinblastine and sacrificed at 5 min thereafter by decapitation.

RESULTS

In the maturation stage of amelogenesis, smooth-ended (SAs; Fig. 6:1a) and ruffle-ended (RAs; Fig. 6:1b) ameloblasts were distributed as bands (Fig. 6:3) across the maturing enamel surface. These two cell types had different morphologies and a different arrangement of junctional complexes. In a previous study,⁶ it was shown that the lack of distal tight junctions allowed peptides of various molecular weights to pass across bands of SAs and enter the enamel via the extracellular fluid; however, this pathway was obstructed by RAs (Fig. 6:2).

The major uptake of ⁴⁵Ca (Fig. 6:4a) and ³³P (Fig. 6:4b) occurred in the maturing enamel in a cyclical manner that reflected the distribution of RAs and SAs. In both cases, the majority of mineral ion uptake appeared in enamel related to RAs.⁷ Staining of Ca in teeth from rats (Figs. 6:4a-e), pigs (Figs. 6:5a-e), and rabbits (Fig. 6:5f) with GBHA, arsenazo III, and calmagite revealed distinct banding and swirling patterns reflecting RA-SA distribution. GBHA stained enamel related to SAs² while the other stains were specific for RA-related enamel.³

Administration of vinblastine caused a gradual loss of RAs such that by 8 hr, only SAs were present in the

maturation stage (Fig. 6:6). These morphological changes correlated with decreasing uptake of Ca into the enamel¹ (Figs. 6:7a-c) and increasing GBHA¹ (Figs. 6:7d-f) and calcein stainability (Figs. 6:7g-i).

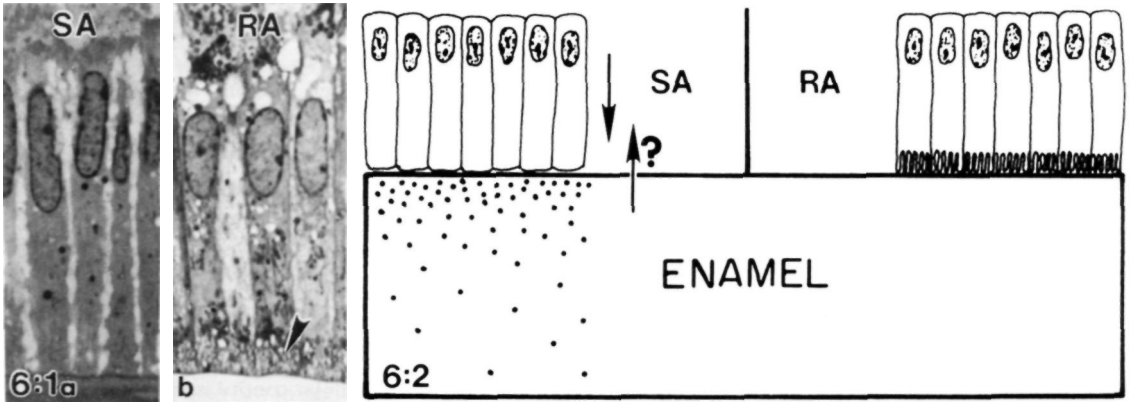


Fig. 6:1. Smooth-ended (SA; a) and ruffle-ended (RA; b) maturation ameloblasts of the rat incisor. RAs are characterized by an extensively infolded distal cell membrane that forms a ruffled border (arrowhead). These cells have distal tight junctions whereas SAs are open to the enamel at their distal ends.

Fig. 6:2. Schematic diagram indicating differences in extracellular permeability between RAs and SAs. The experimental passage of small peptides across SAs and into the enamel, but not across RAs, suggests that this paracellular route may be a pathway for loss of organic matrix degradation products during the maturation stage.

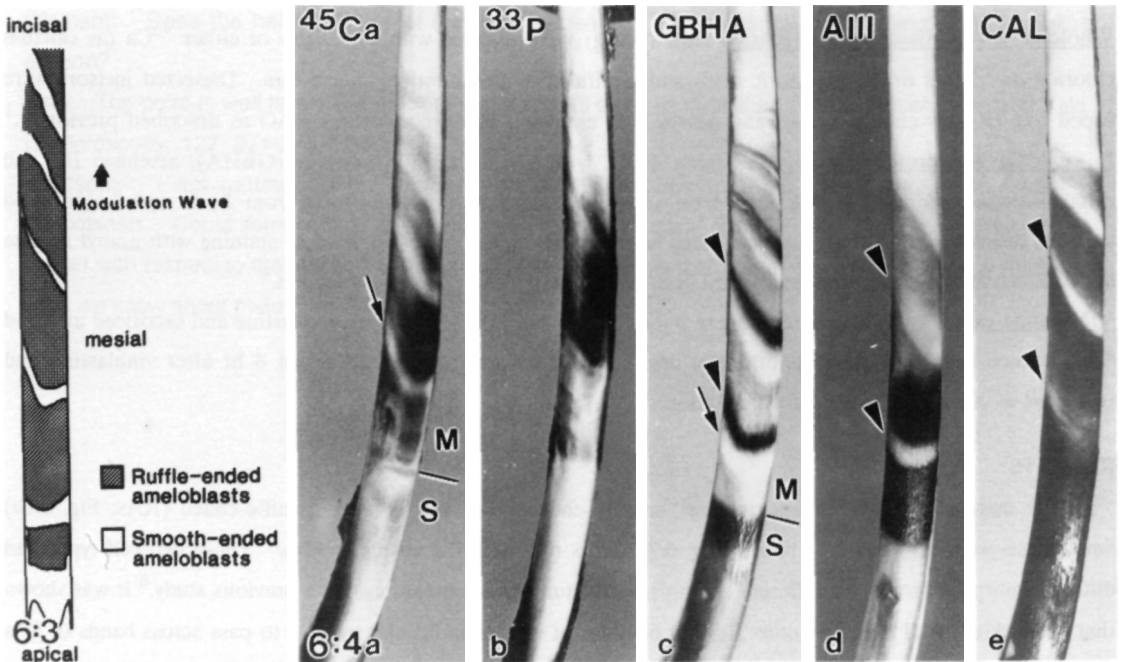


Fig. 6:3. Serial section reconstruction of RAs and SAs in the maturation stage of the lower incisor of the rat. RAs are the predominant cell type and are interrupted by narrow, and often oblique, bands of SAs.

Fig. 6:4. Whole-mount radioautographs of lower incisor enamel from rats sacrificed 5 min after injection of ⁴⁵Ca (a) and ³³P (b). These, and incisors stained with GBHA (c), arsenazo III (d) and calmagite (e), all show banding patterns reflecting RA-SA distribution (compare with Fig. 6:3). The majority of mineral ion uptake is associated with RAs (a,b). Arrows indicate regions corresponding to the newly-forming ruffled border and arrowheads indicate regions corresponding to SAs (see Fig. 6:9). M, maturation stage enamel; S, secretory stage enamel.

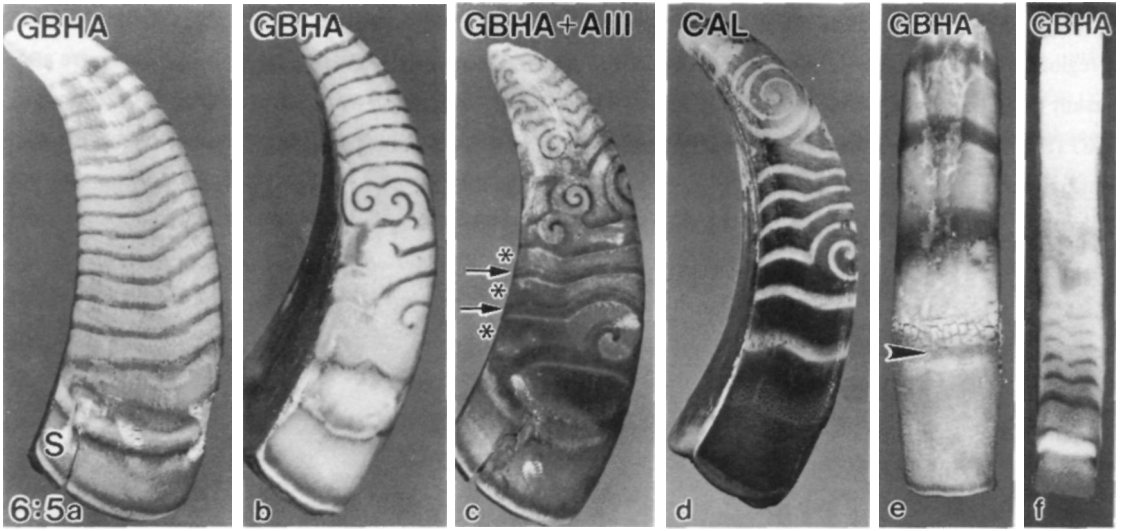


Fig. 6:5. Pig canines (a-d), incisor (e) and rabbit incisor (f) stained with the Ca-complexing agents GBHA arsenazo III (AIII) and calmagite (CAL). Extensive "swirling" is a common feature of the staining pattern found in porcine canines. The staining patterns of teeth first immersed in AIII (asterisks, c), and then counterstained with GBHA (arrows, c), indicate that these two stains identify RA- and SA-related enamel, respectively. S, secretory stage enamel; Arrowhead, junction between secretory and maturation stage enamel.

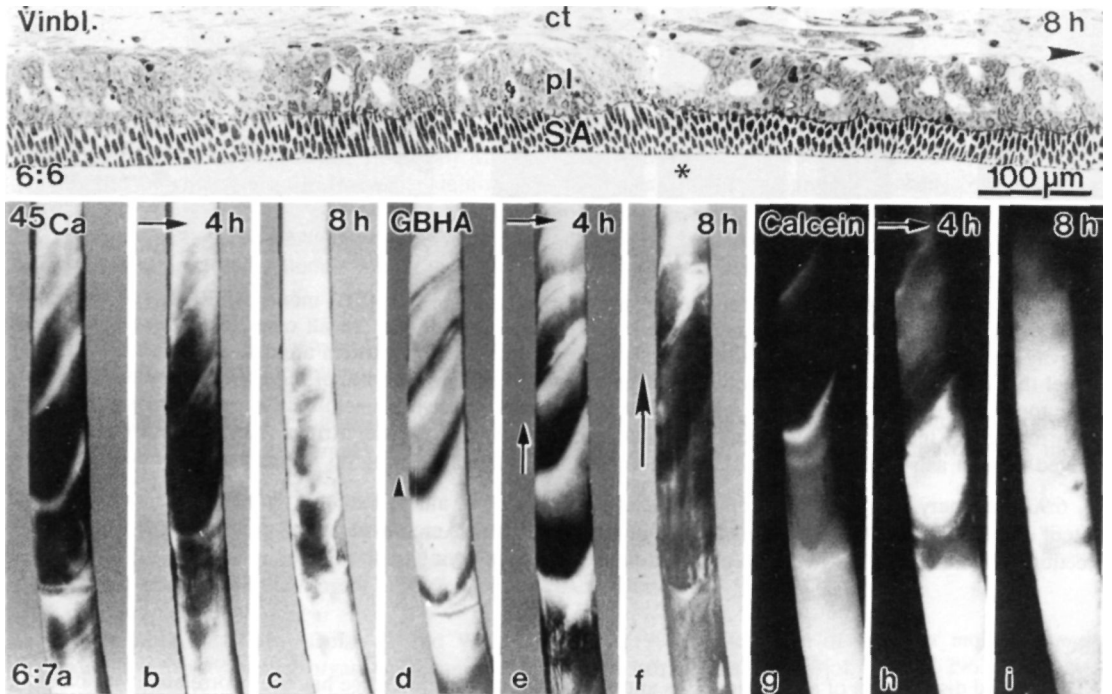


Fig. 6:6. Light micrograph of a segment of a lower rat incisor 8 hr after injection of vinblastine. Only SAs are observed during the maturation stage. ct, periodontal connective tissue; pl, papillary layer; asterisk, enamel space.

Fig. 6:7. Radioautographic and staining patterns in rat maturing enamel at 0, 4, and 8 hr (as indicated) after administration of vinblastine. Radiolabeled Ca uptake diminishes (a-c), and GBHA (d-f) and calcein (g-i) staining increases, at 4 hr after vinblastine. By 8 hr, these effects are maximal as one full wave of RA to SA modulation has propagated across the interband distance (arrows d-f) during this time. The complementary SA to RA conversion has not occurred due to presence of the microtubule-disrupting drug.

Staining for protein content (Figs. 6:8a-c) revealed banded accumulations of protein in the maturing enamel in regions where maturation ameloblasts were forming a new ruffled border. Smaller stripes of protein were also present (Figs. 6:8b,c) and these represented sites of nascent protein secretion as shown at 1 hr by ^{35}S -methionine RAG (Fig. 6:8d).⁴ Fig. 6:9 summarizes the relationships among ameloblast morphologies, Ca entry, enamel protein distribution and GBHA staining of Ca.

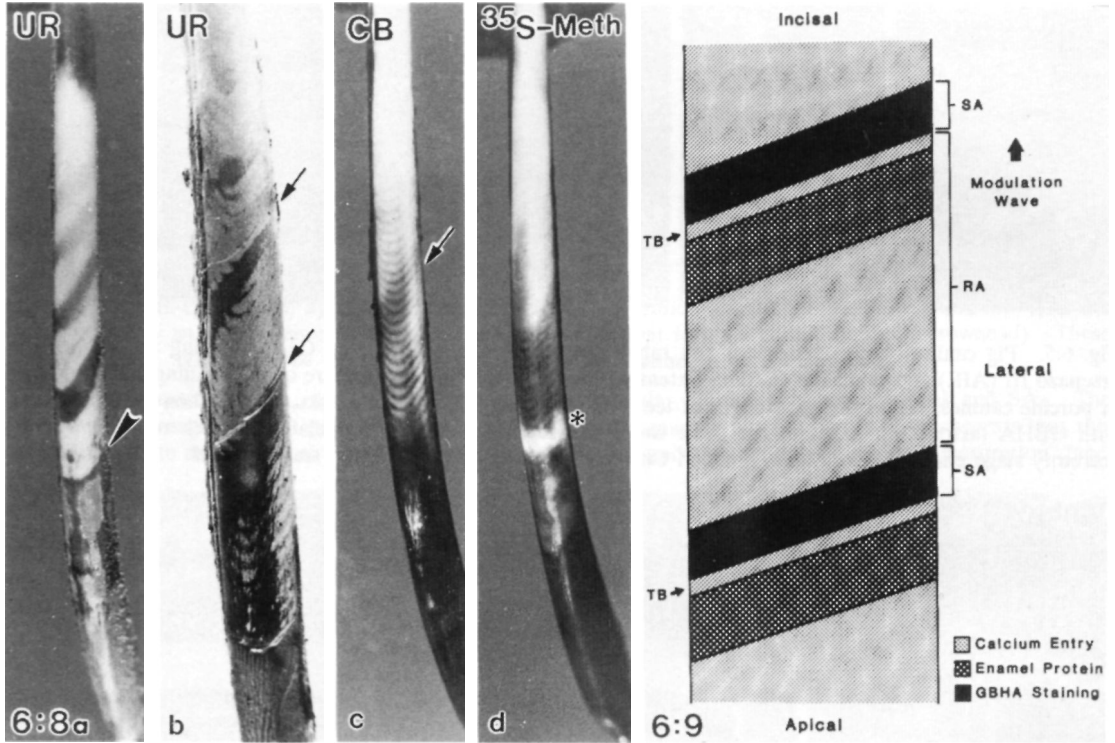


Fig. 6:8. Rat lower incisors stained for protein using uranyl acetate (a,b [BEI mode of SEM]) and coomassie blue (c) and at 1 hr after ^{35}S -methionine injection (d) followed by RAG. In all cases, secretory stage enamel is heavily stained (e.g. below arrowhead in a) or labeled (d) and bands of protein are distributed across maturing enamel that reflected the RA-SA distribution. The arrows in (b) mark the site of the TB observed during drying of the tooth and used to position the protein bands relative to overlying ameloblast morphology (see Fig. 6:9). The arrow in (c) indicates a faint band observed among numerous parallel stripes (b,c) that represent newly-secreted protein as shown by RAG (d). Asterisk, band of enamel showing no radioautographic reaction.

Fig. 6:9. Summary diagram illustrating the relationships among ameloblast morphologies, Ca entry, enamel protein distribution and GBHA staining of Ca in the rat incisor. Ameloblast modulation proceeds in an incisal direction (large arrow). TB, translucent band observed during drying of the tooth.

DISCUSSION

The banded distribution of alternating RAs and SAs in the maturation stage has been correlated with banding patterns in the enamel detected by RAG after injection of radiolabeled mineral ions and after various *in vitro* staining procedures. This correlation indicates that biochemical and physicochemical processes occurring during enamel maturation are closely related to, and probably a direct action of, ameloblast morphology and function. These features of the maturation stage of amelogenesis appear to be common to teeth of continuous and limited eruption from many different mammalian species.

The production of fully-mineralized enamel is ultimately achieved in the maturation stage of amelogenesis. RAs are the major cell type associated with Ca entry into the enamel.⁷ Although the exact nature of this uptake (e.g. isotopic exchange, crystal growth or complexing with organic material) remains to be determined, these cells nevertheless appear to precisely control the addition of this mineral ion to the enamel. One cellular mechanism for such control might reside in the Ca-ATPase pump that is present in the ruffled border membranes of RAs.^{8,9} Not surprisingly, the accumulation and localization of this ion-transporting enzyme occurs in a tight junction delimited membrane domain whose orientation and compartmentalization may be further maintained by subcortical cytoskeletal elements as seen in other polarized epithelial cells. Indeed, drug-induced disruption of the ruffled border membrane arrangement results not only in a loss of Ca uptake into the enamel but in a disturbance of the Ca environment as well (as shown by Ca histochemistry).¹ Therefore, the cyclical changes in cell morphology associated with normal maturation ameloblast modulation⁵ appear to be necessary for the final mineralization of enamel. These cycles likely represent an expression of the physicochemical processes needed to maintain local conditions which are "ideal" for continued deposition of Ca and P into the enamel. The maturation ameloblasts presumably participate via their modulation in creating the extracellular changes necessary for continued increase in the volume of mineral by gradually removing, or allowing passage of, enamel proteins and water from the enamel layer. The net result may be an increase in total mineral content through periodic and short bursts of Ca and P additions by RAs to a specific, cell-defined band (volume) of enamel. It is, therefore, the cumulative time that ameloblasts spend engaged in the maturation stage, and the number of modulation cycles completed by this time, which seem to be important to enamel maturation. Supported by the MRC of Canada and the NIH, USA.

REFERENCES

1. McKee M.D. and Warshawsky H. (1986) Modification of the enamel maturation pattern by vinblastine as revealed by glyoxal bis(2-hydroxyanil) staining and ⁴⁵calcium radioautography. *Histochem.* 86: 141-145.
2. Takano Y., Crenshaw M.A., Bawden J.W., Hammarstrom L. and Lindskog S. (1982) The visualization of the patterns of ameloblast modulation by the glyoxal bis(2-hydroxyanil) staining method. *J. Dent. Res.* 61: 1580-1586.
3. McKee M.D. and Warshawsky H. (1989) Banding patterns in rat incisor enamel stained by histochemical complexing methods for calcium. *Anat. Rec.* 224: 7-13.
4. McKee M.D., Wedlich L., Pompura J.R., Nanci A., Smith C.E. and Warshawsky H. (1988) Demonstration by staining and radioautography of cyclical distributions of protein at the enamel surface in rat incisors. *Archs. Oral Biol.* 33: 413-423.
5. Smith C.E., McKee M.D. and Nanci A. (1987) Cyclic induction and rapid movement of sequential waves of new smooth-ended ameloblast modulation bands in rat incisors as visualized by polychrome fluorescent labelling and GBHA-staining of maturing enamel. *Adv. Dent. Res.* 1: 162-175.
6. McKee M.D., Martineau-Doizé B. and Warshawsky H. (1986) Penetration of different molecular weight proteins into the enamel organ and enamel of the rat incisor. *Archs. Oral Biol.* 31: 287-296.
7. Takano Y., Crenshaw M.A. and Reith E.J. (1982) Correlation of ⁴⁵Ca incorporation with maturation ameloblast morphology in the rat incisor. *Calcif. Tissue Int.* 34: 211-213.
8. Takano Y. and Akai M. (1987) Demonstration of Ca²⁺-ATPase activity in the maturation ameloblast of rat incisor after vascular perfusion. *J. Electron Microsc.* 36: 196-203.
9. Salama A.H., Zaki E. and Eisenmann D.R. (1987) Cytochemical localization of Ca²⁺-Mg²⁺ adenosine triphosphatase in rat incisor ameloblasts during enamel secretion and maturation. *J. Histochem. Cytochem.* 35: 471-482.

Poster 6

McKee: I would like to try and relate some of the events that are happening in the enamel to the cell type which overlies that specific volume of enamel. In the summary diagram (Fig. 6:9) oblique bands of smooth-ended ameloblasts (SA) extend across the tooth and ruffle-ended ameloblasts (RA), the major cell type, extends between the two bands of SA. On this diagram are depicted three events that are occurring in the enamel matrix in relation to these two cell types. One of these events, glyoxal bis (2-hydroxyanil) (GBHA) staining, was first shown in 1982 by Takano et al., (J. Dent. Res. 61: 1580-1586), shows that this particular stain, here shown in black, specifically stains calcium in enamel related to smooth-ended ameloblasts. This demonstrates a very striking banded pattern. The calcium entry pattern, as shown on whole-mount radioautographs, where you can get a broad view of calcium entry into the enamel, also presents a banding pattern that correlates very precisely to this cell distribution. The majority of calcium entry into the enamel is related to ruffle-ended ameloblasts having a well developed ruffled border. At the incisal end of the band of ruffle-ended ameloblasts in the region where the cells are forming a new ruffled border, the signal from the wave of modulation is triggering the formation of a new ruffled border as the cells that were previously SAs become RAs. The enamel adjacent to this newly forming ruffled border is where you find a local accumulation of enamel proteins at the surface of the tooth. The techniques that we use allow us to visualize only the surface of the tooth, so we cannot make any comments with regard to how deep these staining patterns extend toward the dentino-enamel junction. However some work has been done by Takano et al (Archs. Oral Biol. 33: 231-236, 1988.) with GBHA staining, showing that bands of enamel in the early maturation zone are capable of being stained by GBHA throughout the entire depth of the enamel. As you move incisally into more mature enamel these staining events are restricted more and more to just the surface. So we propose that these cyclical events occurring in the enamel result from the same process occurring over and over again, but to different extents depending on which region of the maturation zone you look at. The effect of ameloblast modulation appears to be greatest in the early maturation zone where the major changes take place with regard to protein loss from, and mineral entry into, the enamel.

Deutsch: Have you ever sampled that region of enamel and tried to look with analytical methods to verify whether it is a protein, and if so, how much and what protein it is?

McKee: The banding patterns were obtained by dipping the tooth in protein stains to reveal the distribution of protein at the surface of the incisor. The problem with the rat incisor is that the sample sizes are very small, and hard to dissect. So, we have just recently began some work on the pig incisor which has very broad stained regions (Fig. 6: 5e). We know which cell type corresponds to the stained areas and these pig teeth provide nice sample sizes to work with. We are currently dissecting from the tooth these cell correlated enamel bands and performing biochemical and mineral analyses of these regions.

Bawden: When you say you measured calcium entry under the beginning of the RA band as being heavy, how do you measure net influx of calcium at that point?

McKee: This is the banding pattern you get when you systemically inject calcium⁴⁵ (⁴⁵Ca), sacrifice the animal after 5 mins, dissect the tooth, wipe off the cells, and process the specimen for radioautography.

Bawden: You cannot use a tracer and conclusively draw the conclusion that you have a net influx?

McKee: I did not conclude that this calcium is going immediately toward crystal growth. This is simply the entry pattern as it relates to the two cell types.

Bawden: But it may be not a net influx.

McKee I agree.

Bawden: We have taken enamel from under these bands, (Bawden et al., J. Dent. Res. 67: 938-941, 1988) from cows teeth where the bands move fairly slowly. We simply cannot correlate the calcium content with a banding pattern. Perhaps our method was not sufficiently sophisticated to do that.

McKee: We also attempted that on the rat incisor, and tried a number of biophysical techniques, such as electron probe X-ray microanalysis, X-ray diffraction, X-ray photoelectron spectroscopy (ESCA) and infra-red spectroscopy. These techniques look at the calcium environment in each region and we were also not able to detect any differences, but nevertheless, and although very crude, the staining techniques show banding patterns. These appear to be, at present, the most sensitive way to visualize calcium in different chemical environments.

Bawden: It is a distinction?

McKee: Yes.

Bawden: It may be merely the proportion of the calcium that is free and bound, rather than the total amount that affects your staining.

McKee I agree

Deutsch: What does the GBHA actually stain? If it is ionic calcium, what is the evidence for that ?

McKee: GBHA was the first stain that was used to look at calcium in this fashion by Takano and his co-workers in 1982. We subsequently used GBHA and the other stains (McKee and Warshawsky, Anat. Rec. 224: 7-13, 1989), to show similar banding patterns. Of particular interest is the fact that arsenazo III and calmagite have completely opposite staining patterns to that of GBHA. In a general sense, all these stains are supposed to be specific for calcium under the conditions used. So obviously, there are different states of calcium that these stains are detecting. Unfortunately, the exact chemical binding reactions of each of these stains are still relatively unknown and it is very difficult to extract definitive data about what the calcium environment might be, and that is why we tried some other biophysical methods. All I can say is that calcium appears to be processed somehow in relation to cycling of the organic material and cycling of ameloblast morphology. All these events are tightly coupled and the determination of which comes first is a problem. It is still not certain whether the cells have a programmed timetable whereby they know when to begin modulation and at what rate to propagate the wave, or whether components within the enamel matrix itself are in fact responsible for providing the signal to induce morphological change. Personally, I believe the second possibility is more likely but that has yet to be determined.

Limeback: Up to now we have been used to seeing the modulation patterns occurring in a sequential manner in an incisal direction, usually in oblique bands. Could you speculate as to what the swirl patterns on the porcine canine mean in terms of the final maturation of the enamel?

McKee: I was indeed rather surprised when I pulled these teeth out of the staining solutions. Fig. 6:5a-d, are permanent canines from six month old pigs. These teeth were not yet erupted and you can see

that the majority of the tooth in each case is in the maturation stage. Fig. 6: 5c, is a tooth doubly stained with GBHA and arsenazo III. This swirling phenomenon that you see with GBHA, and inversely with the arsenazo III presumably corresponds to the two cell types, RAs and SAs. The signal whereby the swirling is created must be complex. It was pleasing to see these beautiful patterns. It must be emphasized that the wave of modulation corresponds to a wave of morphological changes. The cells themselves, the ameloblasts, never move in relation to a given volume of enamel. These swirling patterns are not a compression of cells, they are a reflection of a signal whereby the cells change morphology spacially and temporally according to this swirling pattern. I have no doubt that the wave of modulation exists in the pig dentition although the rates may be different and the number of bands may be different.

Deutsch: The only way one could get an idea of absolute uptake of calcium would be to relate the amounts of calcium to tissue volume. If you do a volume analysis, you are going to get an absolute value.

McKee: That is true and that is something we hope to do with the pig incisor enamel.

Robinson: What is GBHA staining? Is it staining calcium under these conditions or an organic component? How does decalcified enamel matrix stain with GBHA? Uptake of radioactive calcium or phosphate and staining with GBHA may reflect matrix removal and not mineral ingress.

McKee: In an attempt to look at what GBHA might be staining, we looked at a number of calcium phosphate phases and stained them with the same procedure we used on the teeth. These results must be "taken with a grain of salt" because any time you do a binding adsorption study, surface areas and other parameters have to be calculated. Nevertheless, hydroxyapatite samples did not stain. The one that stained the darkest red was an amorphous calcium phosphate as determined by X-ray diffraction. This does not answer the question about staining in the rat tooth, but we are hoping to derive these answers from our dissection and analysis of pig incisor enamel in which we are currently analyzing both the organic and mineral components, and trying to detect which ones complex with GBHA.

Takano: The whole amount ^{45}Ca autoradiogram of rat incisors in Dr. McKee's poster shows several bandings of unlabelled areas on the maturing enamel surface. These unlabelled areas appear to correlate with the incisal boundary regions of each RA band in the overlying ameloblast layer. No firm evidence that explains the lack of calcium entry in these particular regions of the enamel has been proposed. In (Fig. 11:9) I would like to show histochemical data that appears to correlate with such an autoradiographic phenomenon. This is a longitudinal Vibratome section of decalcified rat incisor enamel organ at the enamel maturation stage showing the sites of Ca-ATPase activity. Prior to perfusion fixation with glutaraldehyde-paraformaldehyde mixture, the rat was vascularly perfused with a physiological solution to which 5 mM iodoacetamide was added. (Takano and Akai: J. Electron Microsc., 36:196-203, 1987). An intense Ca-ATPase reaction is shown in the ruffled border of RA. It is negative in SA, except for some reactions at the Golgi region. If you look carefully at the ameloblasts from the apical to the incisal direction, you may realise that the reactions associated with the ruffled border gradually decrease and become almost negative at the site corresponding to the region of the enamel where ^{45}Ca labelling is almost negative in the whole amount of the ^{45}Ca autoradiograph. The absence of enzymatic reactions in SA plasma membranes (where ^{45}Ca appears to diffuse paracellularly into the enamel), and the strong reactions in the ruffled border of another group of RA located incisally to the SA band, clearly correlate

with the ^{45}Ca labelling patterns over the maturing enamel surface. The Ca-ATPase thus localised may play a role in the regulation of calcium entry into the maturing enamel of the rat incisor.

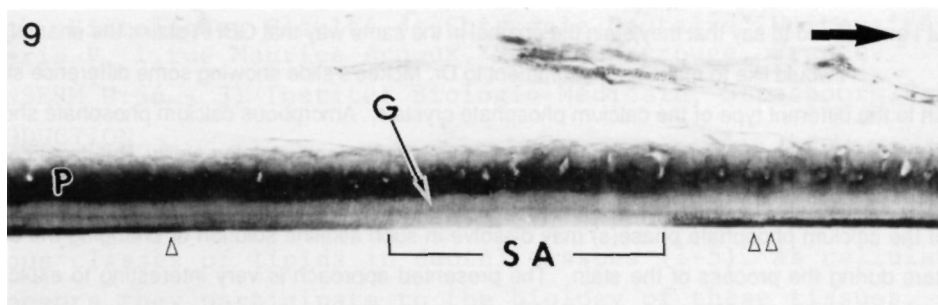


Fig. 11:9. Ca-ATPase reactions in the enamel organ of the vascularly perfused rat incisor (longitudinal Vibratome section). An intense Ca-ATPase reaction (dark stain) along the ruffled border of RA (arrowhead) gradually decreases as it approaches to the incisal Sa band. The ruffled border of the Ra incisal to the SA band show strong enzymatic reactions (double arrowheads). G = weak Ca-ATPase reactions in the Golgi region P = papillary layer Arrow points to the incisal direction.

Aoba: I would like to make some comment to Dr. McKee's demonstration showing some difference staining ability on to the different types of the calcium phosphate crystals. Amorphous calcium phosphate show the strongest staining ability, while hydroxyapatite shows much less, if any, staining ability. But if you consider the process, the GBHA solution has a very high pH. It is assumed that some of the calcium phosphate phase(s) may dissolve in such an alkaline solution or change their surface characteristics during the process of staining. The presented approach is very interesting to explore the mechanism of GBHA stainability on the enamel surface. At the same time we also have to pay attention to the calcium activity or calcium concentration in the solution.

Clement: All the teeth I have seen so far treated in this way are teeth of very rapid growth or even teeth of unrestricted growth. Have similar experiments been done on teeth of limited growth?

McKee: When the first paper on these banding patterns was published by Takano et al in 1982, they showed a number of molars and teeth of limited eruption from other species including the monkey and they all have these banding patterns. What seems to vary is the number of bands. Probably the rate of the wave of modulation varies and the number of modulations that pass over a given volume of enamel varies from species to species.

Bawden: I think that the problem may be that these bands are moving. The effects are blurred. We were using cow enamel where the modulations are moving slowly. But, perhaps not slowly enough.

Sasaki, T.: I imagine that the staining must be mainly on the surface of the enamel matrix?

McKee: Initially, in the early maturation stage enamel, it occurs throughout the full depth of the enamel.

Sasaki, T.: Full depth?

McKee: Yes, but more incisally, the staining is gradually restricted to the surface of the enamel.

Sasaki, T.: Is there any difference among the dyes you used, for example GBHA and Arsenazo III in the thickness of the staining?

McKee: We have not yet checked all of the dyes. I hesitate to put forth at this time to what depth they stain but I am inclined to say that they stain the enamel in the same way that GBHA stains the enamel.

Aoba: I would like to make some comment to Dr. McKee's slide showing some difference staining ability on to the different type of the calcium phosphate crystals. Amorphous calcium phosphate show the strongest staining ability, while hydroxyapatite shows much less, if any, staining ability. But if you consider the process of the stain with GBHA it is GBHA solution has certain pH (a very high pH). It is assumed that some of the calcium phosphate phase(s) may dissolve in such alkaline solution or changing the surface characters during the process of the stain. The presented approach is very interesting to explore the mechanism of GBHA stainability on enamel surface. At the same time we also have to pay attention to the calcium activity or calcium concentration in the solution.