

DEMONSTRATION BY STAINING AND RADIOAUTOGRAPHY OF CYCLICAL DISTRIBUTIONS OF PROTEIN AT THE ENAMEL SURFACE IN RAT INCISORS

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Summary—Staining patterns in the enamel during the maturation stage of amelogenesis reflect the banded distribution of ruffle-ended and smooth-ended ameloblasts. This study investigated the possibility that proteins at the enamel surface may be distributed cyclically according to cyclical changes in ameloblast morphology. Dissected lower rat incisors were wiped free of their enamel organs and immediately immersed in fixative containing one of the following heavy metal and histological stains: uranyl acetate, lead citrate, Coomassie blue, alcian blue and ruthenium red. Other animals were injected with [³⁵S]-methionine to label newly-formed enamel proteins. Their incisors were dissected, the enamel organs were wiped from the enamel surface, and the teeth were processed as whole mounts for radioautography. Teeth stained by heavy metals were also viewed by back-scattered electron imaging. The *in-situ* staining revealed that proteins were distributed in bands and stripes across maturing enamel. Radioautography revealed that the proteins in the stripes were newly-synthesized and secreted into the enamel by certain maturation ameloblasts. We conclude that the enamel organ expresses cyclical activity in part through secretion of proteins.

INTRODUCTION

Ruffle-ended and smooth-ended ameloblasts in the rat incisor enamel organ are arranged as bands that overlie the maturing enamel either transversely or at an oblique angle relative to the long axis of the incisor (Takano and Ozawa, 1980; Reith and Boyde, 1981a; Warshawsky, 1985; Nanci, Slavkin and Smith, 1987). The ameloblasts rapidly modulate between ruffle-ended and smooth-ended phases, and as many as 45 modulations occur over the same area of enamel during maturation (Smith, McKee and Nanci, 1987). The distribution of these cells is indirectly reflected by the characteristic banding pattern observed after staining with glyoxal bis(2-hydroxyanil) (GBHA; Takano *et al.*, 1982a; Josephsen, 1983; McKee and Warshawsky, 1986a, b). Similar cyclical patterns can be visualized in the enamel at any given point in time by many methods including: radioautography (Reith and Boyde, 1981b; Takano, Crenshaw and Reith, 1982b; Reith, Schmid and Boyde, 1984; McKee and Warshawsky, 1986a; McKee, Warshawsky and Nanci, 1987), various molecular-weight probes (McKee, Martineau-Doize and Warshawsky, 1986), tetracycline and fluorochrome labelling (Takano and Ozawa, 1980; Boyde and Reith, 1981; Josephsen, 1983; DenBesten, Crenshaw and Wilson, 1985; Smith *et al.*, 1987), EDTA etching by perfusion (Reith, Boyde and Schmid, 1982), and staining by other histochemical reagents that bind calcium (McKee and Warshawsky, 1987). Similarly, there is a cyclical

distribution of actin related to junctional complexes in maturation ameloblasts (Nishikawa and Josephsen, 1987). These studies have demonstrated that different types of ameloblasts have different functional roles in enamel maturation.

Ultimately, the alternating ameloblast types produce changes in the enamel that are reflected by the characteristic stained maturation pattern. To do this they can produce changes in the organic or inorganic phases of enamel. Cyclical changes in the mineral phase of enamel can be inferred and correlated with ameloblast morphology by using radioautography after ⁴⁵Ca labelling and various histochemical complexing methods for Ca (references as above). On the other hand, reports of cyclical changes in the organic matrix of enamel that can be correlated with ameloblast morphology are scarce (Boyde and Reith, 1982; McKee and Warshawsky, 1986b). As mineral entry and binding occur in a cyclical manner, and as organic matrices are believed to initiate, organize, and direct crystal growth in mineralized tissues (reviewed by Glimcher, 1976; Bonucci, 1984; Veis, 1985; Weiner, 1986), we predicted that certain components of the organic phase of enamel might also be distributed differentially. To investigate this we used various stains to demonstrate the distribution of organic components at the enamel surface; we correlated this distribution with overlying ameloblast morphology, and demonstrated their ability to secrete protein with whole-mount radioautography.

MATERIALS AND METHODS

Animal and tissue handling prior to staining

Male Sherman and Sprague-Dawley rats, weighing 100 ± 20 g, were killed by decapitation under ether

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anaesthesia and the lower incisors were quickly dissected from the alveolar bone. Enamel organs were wiped from the teeth in an apical direction with gauze soaked in ice-cold saline, and wet incisors were transferred immediately to staining solutions. Another set of incisors had the enamel organs removed by this same wiping procedure (identical to that performed on teeth to be stained as described below), while others were on purpose only lightly wiped. To visualize the enamel surface by transmission electron microscopy (TEM) after wiping, these teeth were demineralized in 4.13 per cent EDTA (Warshawsky and Moore, 1967), dehydrated in acetone and embedded in Epon 812 substitute. Thin sections were stained with uranyl acetate and lead citrate, and viewed at 80 kV with a JEOL 2000FX.

Staining procedures

The stains were freshly prepared in fixative containing 2 per cent glutaraldehyde, 0.1 M sodium cacodylate and 0.05 per cent CaCl_2 , and filtered through Whatman no. 50 filter paper. The stains (Sigma Chemical Co., St Louis, Missouri, U.S.A. and JBEM Services, Montreal, Canada) were prepared in fixative as follows: (1) 4.0 per cent uranyl acetate (prepared and used in the dark); (2) 4.0 per cent lead citrate; (3) 0.1 per cent Coomassie brilliant blue G; (4) 0.01 per cent alcian blue 8GX; and (5) 0.01 per cent ruthenium red (ruthenium oxychloride ammoniated). Both incisors from the same animal were stained at room temperature with the same stain for 1-3 h, rinsed vigorously in distilled water and air-dried at room temperature. While drying, some incisors were scored with a scalpel at the positions of the narrow translucent bands (Takano *et al.*, 1982a). Alternatively, some stained teeth were transferred before drying to the same fixative but without the stain; these incisors were soaked for 2-6 h to evaluate the possibility of simple diffusion of stain into the enamel. Other teeth were stained with aqueous solutions without fixative and some incisors were fixed before staining. Paired contralateral incisors were fixed to a platform with Plasticine and photographed. Uranyl acetate-stained teeth were photographed with a Wratten no. 47 blue filter to enhance contrast.

Back-scattered electron imaging (BEI) of stained incisors

The teeth were mounted on aluminum stubs with Plasticine and then carbon-coated. The preparations were examined with a JEOL JSM 840 scanning electron microscope fitted with a LaB_6 cathode and with a JEOL back-scatter divided annular-type detector. Optimum image quality was attained at an accelerating voltage of 10 kV with a 110 μm objective aperture at a working distance of 34 mm with no stage tilt. The back-scattered electron images were recorded directly onto type 55P/N Polaroid film. Inverse signal polarity was used that makes electron back-scattering areas appear dark while non-back-scattering areas appear lighter. This inverse BEI image is similar to that seen with normal light photography, and facilitates comparison.

[^{35}S]-methionine injection and radioautography

Seven male Wistar rats weighing 100 ± 2 g received 0.5 mCi [^{35}S]-L-methionine (sp. act. = 1134Ci/mMol;

Dupont NEN Research Products, Boston, Massachusetts, U.S.A.) in 0.2 ml PBS, pH 7.4, via the external jugular vein. Six minutes after injection of the radioactive tracer, the animals received a large excess (10 mM) of non-radioactive L-methionine (Sigma Chemicals) in 0.2 ml PBS, pH 7.4, through the contralateral jugular vein. One hour after the initial injection, the animals were killed by decapitation, the lower incisors dissected and the enamel organs removed by wiping. Both teeth were immediately immersed in 2 per cent glutaraldehyde in 0.1 M sodium-cacodylate buffer, pH 7.4, for 30 min at room temperature. The incisors were washed vigorously in distilled water for several minutes and air-dried at room temperature. They were dipped in a 0.5 per cent gelatin solution containing 0.05 g chrome alum as a hardener for 20 s at 40°C, dipped in Kodak NTB2 liquid emulsion, exposed for 14 days, and developed according to the method of Kopriwa and Leblond (1962). Radioautographic patterns were recorded by normal light photography and by BEI of the silver in the developed radioautographic emulsion as described previously (McKee *et al.*, 1987). Some incisors were stained with GBHA prior to processing for radioautography to correlate ameloblast morphology with the radioautographic pattern. This generally was unsuccessful as the GBHA faded over the 14 days exposure time and was further leached from the enamel during radioautographic development. However, one tooth retained a faintly-stained band in the early maturation zone.

Protein extractions

Eleven male Wistar rats weighing 104 ± 3 g were anaesthetized with ether and decapitated. The labial alveolar bone surrounding the lower incisors was removed and the cells of the enamel organ and associated connective tissues were wiped from the enamel surface in an apical direction using coarse gauze soaked with ice-cold 0.9 per cent saline solution. Pieces of enamel 1 mm in length were separated from the dentine sequentially in an apical and incisal direction from a reference line reflected as a tangent to the labial side of the incisor from the junction between first and second molars (Smith *et al.*, 1987). Each piece of enamel was placed in a separate vial and stored frozen at -20°C . The enamel pieces were thawed to room temperature and each piece was crushed into a fine powder with a blunt dental tool. In one set of experiments (16 teeth), 0.4 ml of 0.5 M NaOH was added to the crushed enamel in each vial. The vials were vortexed, sonicated for 2 min, and immersed for 20 min in a boiling water bath. The vials were cooled and the fluids transferred to standard glass test tubes and assayed for protein against a bovine serum albumin standard using the Lowry analysis (Lowry *et al.*, 1951). In another set of experiments (6 teeth), the vials and a 0.9 per cent saline solution were first equilibrated to 4°C or room temperature (20°C), then 0.1 ml of the saline was added to each vial and the crushed enamel pieces soaked at the given temperature for 1 h. Each vial was centrifuged at maximum setting for 5 min in an Eppendorf microfuge (at the appropriate temperature), and the fluids were decanted and placed in individual test tubes. To each tube 0.1 ml of

1 M NaOH was added followed by 0.2 ml of 0.5 M NaOH. The fluids were then boiled and analysed for protein as described above.

RESULTS

Staining patterns

A clear, cyclical pattern was produced by staining the enamel with uranyl acetate, lead citrate, coomassie blue, alcian blue and ruthenium red. Simple aqueous solutions of the stains revealed the staining patterns but the most distinct results were obtained when stains were dissolved in fixative solutions and the results reported below describe staining in sodium cacodylate-buffered glutaraldehyde.

Incisors stained with uranyl acetate for 3 h at room temperature showed darkly-stained enamel in the secretion zone and 4 or 5 narrow stained bands in the maturation zone (Plate Fig. 1). Shorter time intervals produced the same pattern but less intensely. Most of the bands were obliquely orientated relative to the long axis of the incisor. Soaking fresh incisors for 1 h in physiological saline at room temperature before staining with uranyl acetate in fixative abolished the staining reaction (Plate Fig. 2). To investigate the possibility that the loss of stainability might be due to diffusion of proteins out of the enamel and into the saline, Lowry protein analysis was performed. The supernatant saline fluids, previously containing crushed pieces of enamel soaked for 1 h at 4°C, were compared to the total protein content of similar pieces of enamel boiled for 20 min in 0.5 M NaOH (Text Fig. 12). It was found that by 1 h, approx. 75 per cent of the enamel proteins had diffused out of the enamel and into the saline at room temperature. Pieces of enamel soaked at 4°C for 1 h lost only 20 per cent of their proteins. Consequently, incisors soaked for 1 h at 4°C in normal saline showed the typical pattern, although the staining was slightly diminished. To investigate the alternative possibility that stainability might in part be due to organic debris left at the enamel surface following the wiping procedure, we used transmission electron microscopy of demineralized, longitudinally-sectioned incisors and verified that the enamel surface was free of cellular and other organic material (Plate Fig. 10). The outermost enamel showed no significant damage other than the possibility that a thin layer of enamel might have been removed by the wiping. Incisors that had been only lightly wiped showed some extraneous material at the enamel surface (Plate Fig. 11).

BEI of uranyl-stained teeth relied on the back-scattered signal produced by the high atomic number of uranium ($Z = 92$), relative to the background back-scattering caused by Ca ($Z = 20$) and P ($Z = 15$). BEI of the same teeth shown in Fig. 1 demonstrated the distribution of uranium in 4 narrow bands (Plate Fig. 3). During drying, after staining with uranyl acetate, the enamel showed narrow translucent bands which appeared to dry more slowly than adjacent areas. The incisors had been scored along these translucent bands and BEI revealed that the bands stained by uranyl acetate were located just apical to the score marks (Plate Fig. 4). Thin parallel stripes were frequently observed in the maturation

zone; these followed the orientation of the bands laterally but lost this orientation mesially as the stripes hooked abruptly incisally (Fig. 4). The stripes were most obvious in the first few interband regions.

Incisors stained for 3 h at room temperature with lead citrate showed a pattern similar to that with uranyl acetate (Plate Fig. 5). As lead citrate did not produce a colour reaction suitable for normal light photography, BEI was employed to visualize the pattern; taking advantage of the high atomic number of lead ($Z = 82$). The enamel maturation pattern again consisted of 4 to 5 darkly-stained narrow bands and numerous thin parallel stripes (Fig. 5). Scoring of the translucent bands during drying showed that the stained bands were immediately apical to the score marks.

Staining with Coomassie blue (Plate Fig. 6) and alcian blue (Plate Fig. 7) for 2 h at room temperature was more effective in showing the repeated parallel thin stripes. These stripes began adjacent to the darkly-stained secretion zone enamel and continued along the entire maturation zone, decreasing in intensity as they approached the pigmented enamel. The stripes maintained the same orientation throughout each tooth. In addition, faint staining of the narrow bands was superimposed on the stripes. The narrow bands were orientated differently from the thin stripes and the pattern of the narrow bands was similar to that seen after staining with uranyl and lead. Observation of Coomassie blue-stained and alcian blue-stained incisors while drying revealed that the bands were apical to the translucent bands; in the same position as seen after uranyl and lead staining.

Incisors stained with ruthenium red for 1 h at room temperature also showed a pattern consisting of 4 or

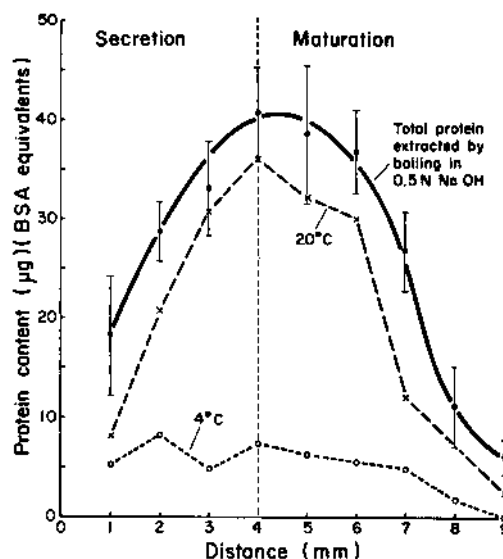


Fig. 12. Graph illustrating the diffusibility of enamel protein during a 1 h soak in physiological saline. The extent of protein removal from the enamel as determined by Lowry assay of the saline supernatant after soaking performed at 4°C (open circles) and 20°C (x) is compared to total protein content obtained by boiling in 0.5 M NaOH (solid circles). Most of the enamel protein diffuses freely out of the enamel at 1 h at room temperature relative to that observed at 4°C.

5 narrow bands of stained enamel in the maturation zone (Plate Fig. 8). These bands were orientated as previously described, but their position as seen during drying was just incisal to the translucent bands, unlike the other stains. The narrow bands decreased in staining intensity incisally. The enamel in the secretion zone was heavily stained and the pigmented enamel did not stain.

In all cases, and with each stain, additional and prolonged soaking in fixative without stain did not diminish, alter or remove the staining pattern. Additionally, soaking of fresh incisors in physiological saline for 1 h at room temperature practically eliminated the ability of the enamel to stain with each of the above-mentioned staining reagents.

Radioautography after [³⁵S]-methionine injection

Whole-mount radioautography 1 h after [³⁵S]-methionine injection showed a repetitive distribution of radioactivity at the surface of the tooth (Plate Fig. 9). Most prominent in this pattern was the presence of thin stripes in the early maturation zone. The enamel in the secretion zone was also heavily labelled. Unlabelled areas in the early maturation zone may correspond to regions of enamel normally overlaid by smooth-ended ameloblasts.

DISCUSSION

Staining reactions

These stains are commonly used to reveal proteins in histological sections and in polyacrylamide gels. They were selected either for their ability to produce a colour reaction when bound to proteins or for their high atomic number, thus making the staining patterns suitable for BEI (McKee *et al.*, 1987). The reactions represent true binding of stain to protein as the staining patterns were not diminished or altered during prolonged immersion in aqueous fixative solutions without the staining agent, and because the stainability of the enamel was lost when unfixed teeth were soaked in saline which extracts protein. The question of whether the stains are coupled directly to the protein or via the fixative was resolved as staining also occurred in aqueous solutions, thus demonstrating that direct complexing occurs between the dye and the protein. The staining patterns represent either surface reactions or penetration of the stains into the enamel; consequently the staining intensity at the surface may be due to the degree and depth of stain penetration. In the case of GBHA staining, we have found in preliminary work that the apical GBHA-stained bands extended as deep as the dentino-enamel junction but the incisal GBHA-stained bands were only at the surface (data not shown). With the stains used here, only surfaces were visualized, and all interpretations are restricted to events occurring at the enamel surface.

Most heavy metal ions form coordination complexes with chemical groups having nitrogen, oxygen, sulphur or phosphorus atoms (reviewed by Zobel and Beer, 1965). Watson (1958a) tested various uranium salts and found that uranyl acetate gave superior results as an electron-dense stain. Uranyl acetate is commonly used as a histological stain: it has a high affinity for the phosphate groups of nucleic acids

(Huxley and Zubay, 1961; Zobel and Beer, 1965) and slightly less affinity for the carboxyl (Stoeckenius, 1961) and free amino (Lombardi *et al.*, 1971) groups of proteins. Based on the binding abilities of uranyl acetate, and because cellular components of the enamel organ were removed before staining, we conclude that staining reveals proteins and that these proteins are distributed in a pattern resembling that given by GBHA. The narrow bands of uranyl-stained enamel were just apical to the translucent bands observed during drying, a region overlaid by ruffle-ended ameloblasts *in vivo* (Takano *et al.*, 1982; Smith *et al.*, 1987). The thin uranyl-stained stripes were found throughout most of the maturation zone in areas that would normally be overlaid by either ruffle-ended or smooth-ended ameloblasts.

Staining with lead was introduced by Watson (1985b); it preferentially stains RNA (Dalton and Zeigel, 1960) and carbohydrates (Reynolds, 1963). Staining may be due to polymeric cations arising from complex lead compounds formed by divalent lead salts in alkaline solutions (Reynolds, 1963). The staining of carbohydrates may depend upon the formation of a stable lead-carbohydrate complex through hydrogen bonding (Reynolds, 1963). The visualization and distribution of the lead-stained pattern in enamel by BEI was identical to that following uranyl staining, and also reflects a differential distribution of protein at the tooth surface.

Coomassie blue is widely used for the demonstration of protein in acrylamide gels (Diezel, Kopperschlager and Hofmann, 1972; Fishbein, 1972) and appears to bind solely and reliably to protein. Furthermore, good results have been obtained using Coomassie blue for the staining of proteins in histological sections (Feder and O'Brien, 1968; Fisher, 1968; Cawood, Potter and Dickson, 1978). Whereas little is known about the binding of Coomassie blue to protein, all Coomassie blue-stained biological material is sensitive to non-specific protease (Cawood *et al.*, 1978), indicating the protein nature of that material. The more intense staining of the thin stripes than the narrow bands of the enamel-maturation pattern indicates a greater accumulation of certain proteins in the surface stripes than in the bands. The position of the translucent bands observed during drying of the Coomassie blue-stained teeth indicates that the narrow bands of stained enamel correspond to regions overlaid by ruffle-ended ameloblasts, whereas the stained thin stripes correlate with both types of ameloblasts.

Alcian blue is a cationic dye used to demonstrate glycosaminoglycans (GAG) associated with proteoglycans. Conventional methods of fixation eliminate or translocate GAG (reviewed by Goldberg, Septier and Escaig-Haye, 1987); however, the addition of a cationic dye to the fixative reduces the mobility, and precipitates and retains these components *in situ* (Scott, 1972). The positive charges of alcian blue react via salt linkages with the negative electrostatic field of complex sugar side-chains to produce insoluble precipitates (Scott, Quintarelli and Dellovo, 1964). Alcian blue staining in fixative solution prevented extraction and translocation of organic enamel components. It revealed a faint staining

of the narrow bands, but an intense staining of the thin stripes associated with the enamel maturation pattern. The staining pattern with alcian blue was practically identical to that after staining with Coomassie blue, indicating that the protein in the thin stripes and narrow bands may have numerous sugar side-chains. This is supported by the similarity of the pattern with that found after staining with the periodic acid-Schiff reaction (McKee and Warshawsky, 1986b) used to visualize glycoproteins.

Ruthenium red is an inorganic cationic dye used in fixatives to demonstrate glycoconjugates (reviewed by Luft, 1971a, b; Goldberg and Septier, 1986). Ruthenium red also precipitates a large variety of polyanions by ionic interaction and its reaction with GAG is typical rather than specific (Luft, 1971a). The pattern observed after ruthenium red staining was different in that the stained areas were incisal to the translucent bands (in areas normally overlaid by smooth-ended ameloblasts). In this regard it was the only reagent that stained in a pattern similar to the Ca stain, GBHA. The reason for the dramatic difference between the staining patterns after ruthenium red and the similar cationic stain alcian blue is not clear. In any case, it appears that each stain is selective for specific organic constituents of enamel that relate to specific and different types of maturation ameloblasts.

Radioautography

Radioautographs of sections from fixed and routinely processed incisors after injection of radio-labelled methionine have revealed that this amino acid is incorporated into proteins that are secreted in both the secretion and maturation zones of amelogenesis (Nanci *et al.*, 1987). Whole-mount radioautography as we used, confirms the presence of material containing [³⁵S]-methionine at, or close to, the enamel surface, and further demonstrates a pattern in the maturation zone consisting of thin labelled stripes representing newly-secreted protein at the enamel surface.

Correlation between staining and radioautography

Our staining patterns demonstrate that protein is differentially distributed at the tooth surface. Biochemical investigations have shown that the organic matrix of enamel contains glycoproteins (Stack, 1954, 1956; Elgyedi and Stack, 1956; Seyer and Glimcher, 1969; Elwood and Apostolopoulos, 1975; Belcourt and Gillmeth, 1979; Belcourt, Fincham and Termine, 1982). By histochemistry and radioautography, glycoproteins have been identified in the enamel (Scheinmann, Weinreb and Wolman, 1962; Suga and Gustafson, 1963; Reith and Butcher, 1967; Weinstock and Leblond, 1971; Goldberg, Genotelle-Septier and Weill, 1979; Warshawsky, 1979; Warshawsky and Josephsen, 1981; Goldberg and Septier, 1986; McKee and Warshawsky, 1986b). Similar histochemical and radioautographic identification of GAG in the enamel have been reported (Belanger, 1955; Bevelander and Johnson, 1955; Leblond, Belanger and Greulich, 1955; Kennedy and Kennedy, 1957; Quintarelli and Dellova, 1963; Clark, Smith and Davidson, 1965; Weill and Tassin, 1965; Lennox and Provenza, 1970; Yoshiki and Umeda,

1972; Nagai and Takuma, 1973; Blumen and Merzel, 1976; Goldberg *et al.*, 1976, 1978, 1979; Nakata *et al.*, 1982; Goldberg and Septier, 1986). Several of these studies localized these molecules ultrastructurally within sections of enamel. Our study has visualized proteins in general, and possibly glycoconjugates, at the surface of rat incisor enamel. The rapid dissection and staining in fixative allows simultaneous fixation and whole-mount staining before the enamel proteins have a chance to translocate significantly. The dissection procedure must be performed rapidly because unfixed enamel proteins rapidly diffuse out of the enamel (Warshawsky, 1985; see Fig. 12). We obtained further evidence that proteins diffuse out of the enamel by abolishing staining in teeth soaked in normal saline before staining (Fig. 2). The protein assay values, determined biochemically after soaking crushed enamel pieces (Fig. 12), are most likely higher than those that would be obtained for diffusion of proteins from the intact enamel layer of incisors soaked in saline. Indeed, soaking of uncrushed enamel pieces results in less diffusion of protein out of the enamel. Nevertheless, we conclude that with time there is enough diffusion of enamel proteins out of the enamel to prevent staining at the tooth surface.

Interpretation of staining patterns

The cyclical nature of enamel maturation with regard to the inorganic phase is well-documented. Staining of certain organic constituents of enamel, and synthesis and release of newly-formed [³⁵S]-methionine-containing proteins by maturation zone ameloblasts, also occurs in a repetitive manner. These observations emphasize that cyclical activity is also related to the organic phase in the maturation zone of amelogenesis. Furthermore, correlations with overlying ameloblast morphology are made possible by GBHA staining (Takano *et al.*, 1982a; Smith *et al.*, 1987) and by scoring the translucent bands during drying (Takano *et al.*, 1982a). These techniques indicate that the maturation pattern is the functional consequence of the rapid modulation of maturation ameloblasts between ruffle-ended and smooth-ended types (Smith *et al.*, 1987). This modulation affects the distribution of the mineral and matrix phases, and interference with the wave of modulation by vinctin (which prevents smooth-ended ameloblasts from reverting back to ruffle-ended ameloblasts) alters the pattern seen in the enamel (McKee and Warshawsky, 1986a). Thus, it is important to determine the significance and relationship between bands and stripes of organic components on the one hand, and bands and stripes of different Ca states on the other.

We found that the enamel surface in the secretion zone was heavily labelled after injection of [³⁵S]-methionine, reflecting the massive secretion of nascent proteins during this stage. Consequently, the surface in the secretory stage stained darkly with all our methods. Radioautography after ⁴⁵Ca injection (Reith and Boyde, 1981b; Takano *et al.*, 1982b; Reith *et al.*, 1984; McKee and Warshawsky, 1986a; McKee *et al.*, 1987) and staining with reagents that complex different states of Ca (McKee and Warshawsky, 1987) show with one exception (arsenazo III), a heavy

reaction in the secretion zone enamel. The colocalization of organic and inorganic events in a region undergoing continuous secretion and mineralization strongly emphasizes the link between matrix and mineral during amelogenesis. It is conceivable that cyclical organic-inorganic events may also occur in the secretion zone, but these may be masked by the secretory activity which comprises the main function at this stage of amelogenesis. Once secretory activity is diminished and the entire layer of enamel is formed at the end of the secretion zone, only maturation events persist and the ameloblast minimizes the secretory aspects while maximizing the maturation aspects. Is is of particular interest that the ruthenium red staining pattern was different in that it stained secretory zone enamel like the other protein stains, but stained maturing enamel only in those regions also stained by GBHA (associated with smooth-ended ameloblasts). Similarly, GBHA staining of the enamel appears to be the inverse of the reactions observed after staining with other Ca stains (McKee and Warshawsky, 1987). Consequently, enamel related to smooth-ended ameloblasts appears to have a matrix-mineral relationship significantly different from adjacent areas related to ruffle-ended ameloblasts.

Our purpose was to demonstrate that there is a cyclical distribution of organic material related to ameloblast modulation at the enamel surface. Some of this material is synthesized and secreted by maturation ameloblasts and may, in part, be glycoconjugates that are known to exist in maturing enamel. We suggest that certain enamel proteins, and regional accumulations of these proteins found within the bands and stripes, contribute to the cyclical processing of Ca prior to its incorporation into the hydroxyapatite of enamel crystallites.

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Plate 1

Fig. 1. Contralateral lower rat incisors wiped free of their enamel organs and stained with uranyl acetate in fixative for 3 h. The enamel in the secretion zone is darkly-stained and, in the maturation zone, 4 narrow bands of stained enamel run obliquely across the teeth. Staining intensity decreases incisally. x 6.5

Fig. 2. An incisor soaked for 1 h in physiological saline at room temperature before staining with uranyl acetate as in Fig. 1. Note the abolition of the staining pattern due to diffusion of enamel proteins into the saline (see Fig. 12). x 6.5

Fig. 3. Inverse back-scattered electron image of the same teeth shown in Fig. 1. This electron-detection mode relies on the relatively high atomic number of the uranium in the stained areas of enamel relative to the background back-scattering produced by the Ca and P in the unstained areas. Again, the presence of 4 narrow bands of stained enamel can be observed, x 12

Fig. 4. Inverse back-scattered electron image of a different pair of teeth stained with uranyl acetate. In this animal, the thin stained stripes (small arrows) are prominent. Observation and direct scoring of the translucent bands during drying of the teeth (arrowheads) indicates that the uranyl-stained narrow bands (as opposed to the thin stripes) are found in regions of enamel normally overlaid by ruffle-ended ameloblasts, x 12

Fig. 5. Inverse back-scattered electron image of incisors stained with lead citrate in fixative for 3 h at room temperature. The staining pattern again consists of 4 to 5 narrow, darkly-stained bands just apical to the scored translucent bands (arrowheads), and thinner parallel stripes (small arrows), x 12

Plate 2

Fig. 6. Contralateral lower incisors stained with Coomassie blue in fixative for 2 h at room temperature. The enamel in the secretion zone is darkly-stained, and in the maturation zone the staining pattern consists of narrow bands (white arrows) and thin stripes (black arrows). This stain is particularly effective in demonstrating the stripes which continue without changing orientation along the entire maturation zone. x 6.5

Fig. 7. Alcian blue staining of incisors shows a pattern similar to that after staining with Coomassie blue. The pattern consists of bands (white arrows) and stripes (black arrows) of stained enamel in the maturation zone. In the secretion zone, the enamel is darkly stained. Staining intensity diminishes in an incisal direction, x 6.5

Fig. 8. Ruthenium red staining of incisors also shows a banding pattern at the surface of the teeth. The pattern consists of 4 narrow bands of stained enamel running transversely and obliquely across the enamel in the maturation zone. The enamel in the secretion zone is intensely stained. The position of the bands with this stain is different from the other stains in that these areas of enamel are incisal to the translucent bands and are consequently overlaid by smooth-ended ameloblasts, x 6.5

Fig. 9. Radioautographs of the low incisors of an animal that received [³⁵S]-methionine 1 h prior to death. The enamel in the secretion zone is heavily labelled and in the maturation zone, thin parallel stripes (black arrows) of radioactivity represent incorporation of [³⁵S]-methionine into newly-synthesized enamel protein released at the surface of the tooth. Unlabelled areas (white arrows) may correspond to enamel related to smooth-ended ameloblasts, x 6.5

Plate 3

Fig. 10. Electron micrograph of the enamel surface of a demineralized incisor wiped free of its enamel organ as in Figs 1-9. Although it is possible that a thin layer of enamel may have been removed during the wiping procedure, the relative cleanliness of this surface indicates that cellular or other organic debris do not contribute to the staining pattern, x 10,250

Fig. 11. Electron micrograph of the enamel surface from a demineralized incisor purposefully wiped only lightly relative to the enamel surface shown in Fig. 10. Some extraneous material remains at the enamel surface (arrows), x 10,250

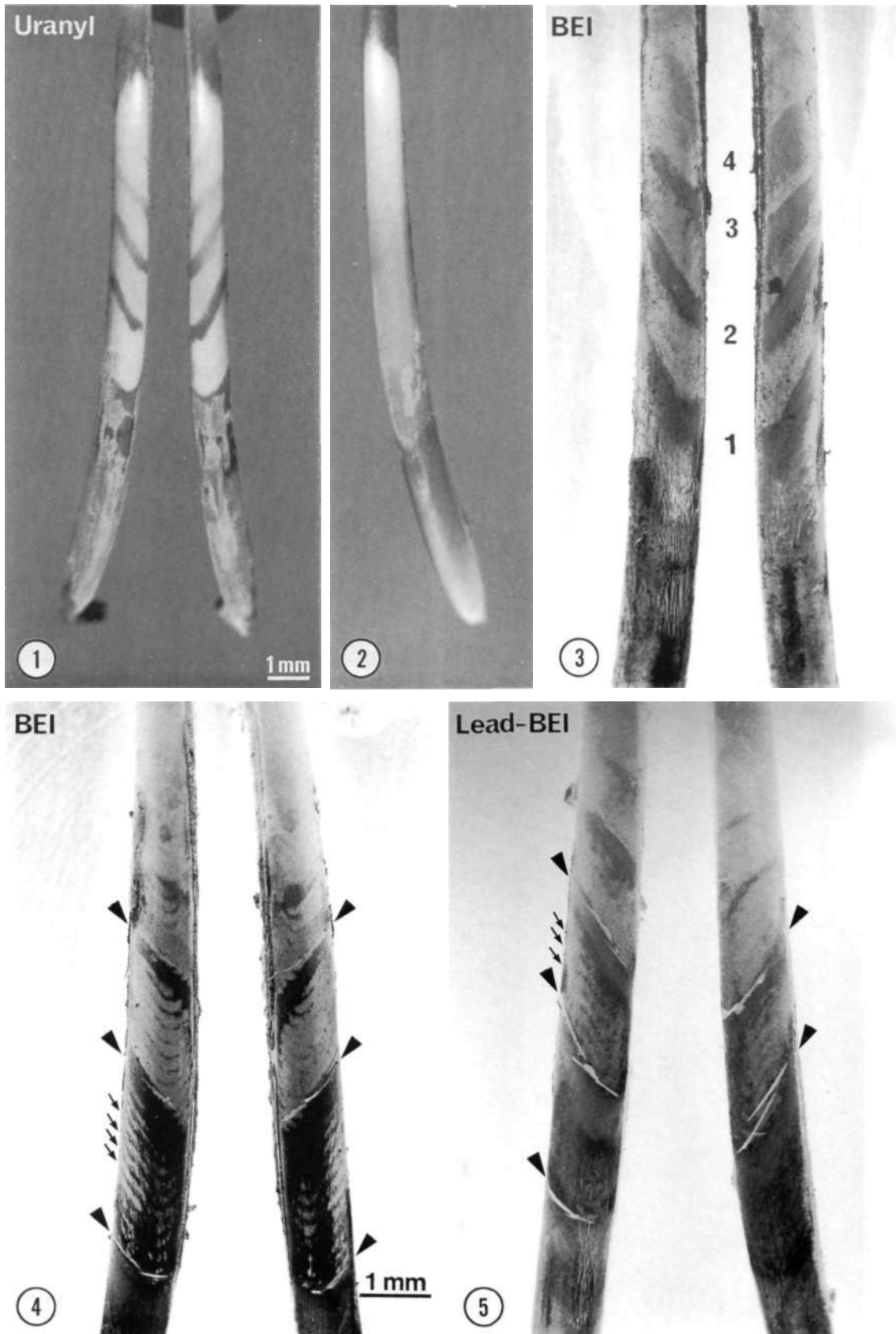


Plate 1

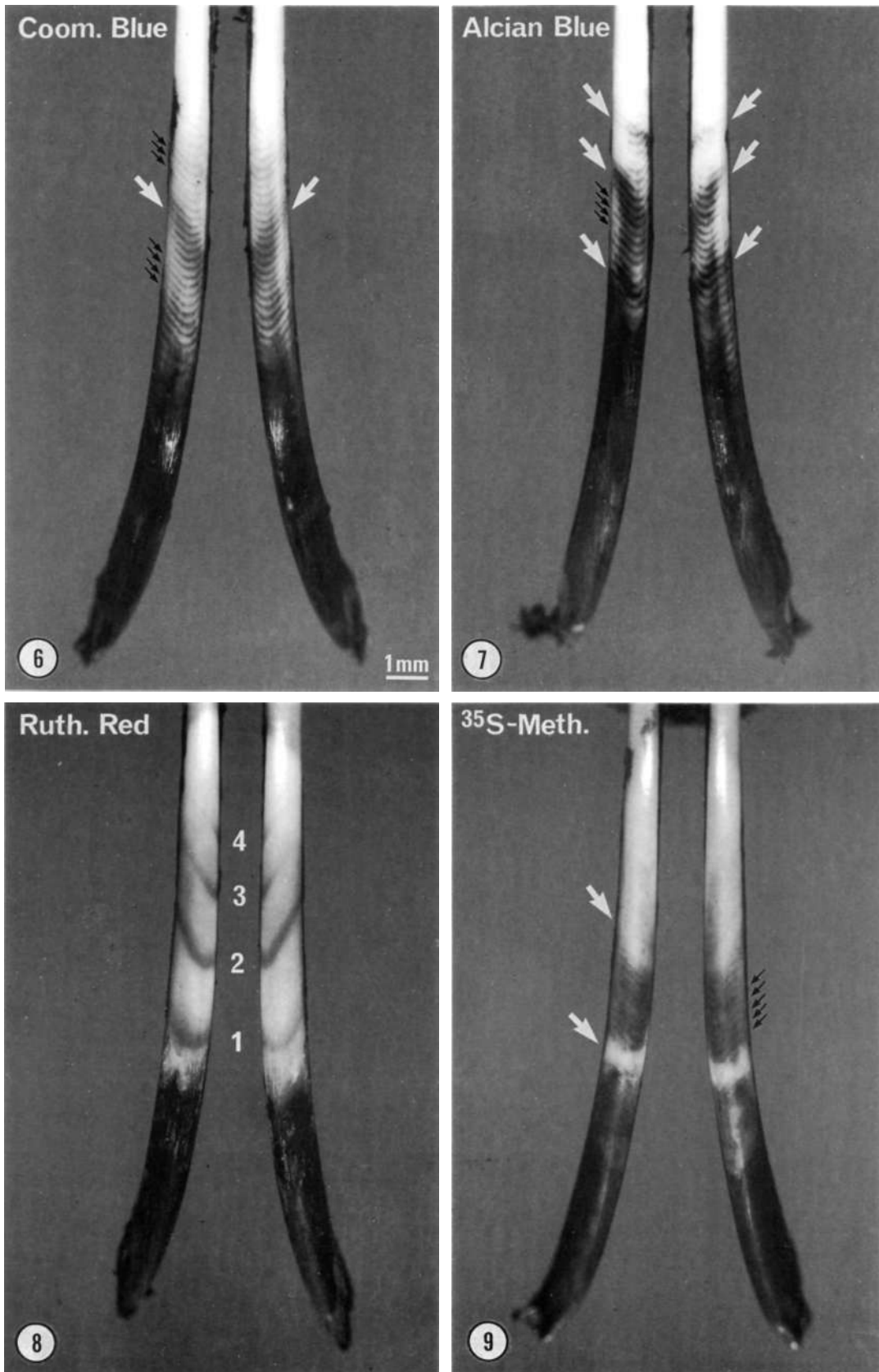


Plate 2

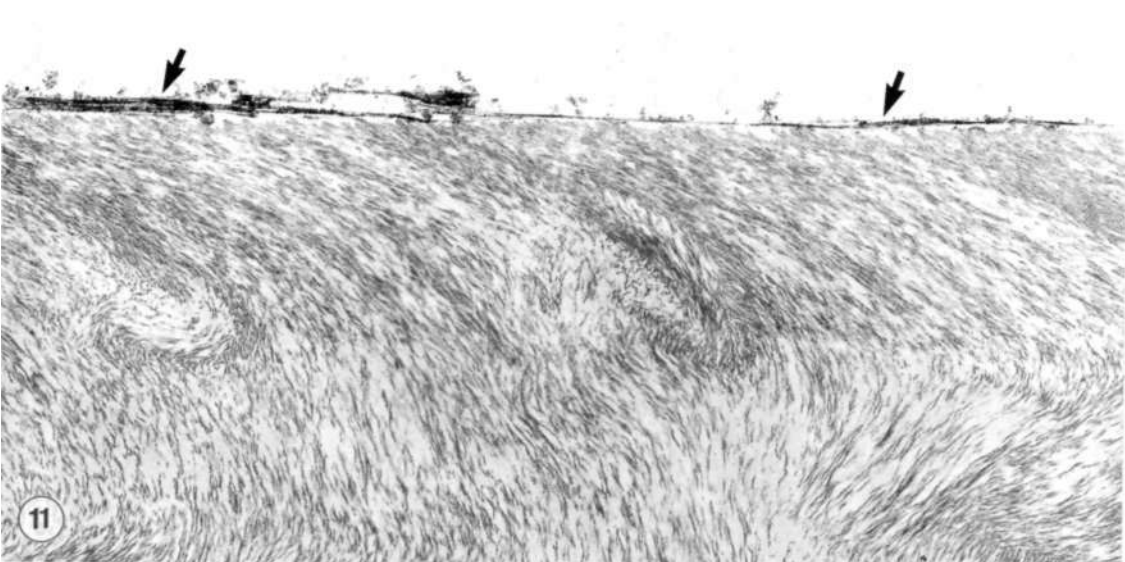
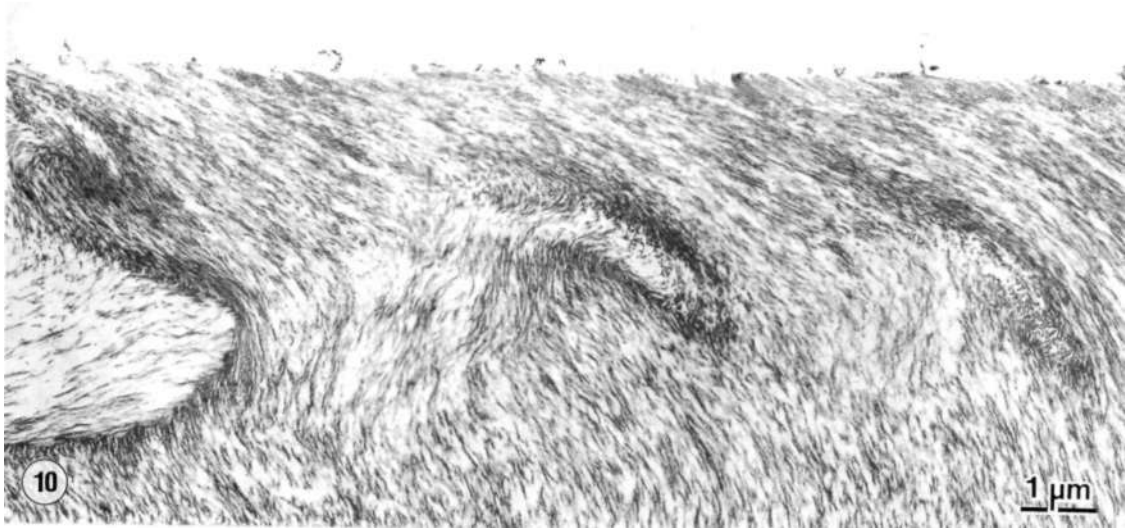


Plate 3