

Development and Calcification of Enamel*

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***Supported by the Medical Research Council of Canada**

**Reprinted from CALCIFICATION IN BIOLOGICAL SYSTEMS,
CRC Press, Boca Raton, FL**

Chapter 13

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I. INTRODUCTION

Enamel was one of the first biological tissues to be examined in the light microscope¹ and has since fascinated and mystified researchers with its complexity. The creation of this tissue exemplifies many fundamental cellular events such as proliferation and differentiation, protein synthesis and secretion, post-translational processing, endocytosis, lysosomal protein targeting and degradation, ion (calcium) transport, and mineralization. There are also several unusual cellular events associated with amelogenesis such as (1) reversal of secretory polarity by ameloblasts; (2) formation and destruction, and eventual recreation of a basal lamina along a surface that constitutes the embryonic bases of preameloblasts but later occupies the functional apices of differentiated ameloblasts; and (3) bulk destruction of almost all of the proteins secreted by these cells. A comprehensive understanding of these phenomena is essential in understanding how enamel forms and mineralizes.

Amelogenesis has been described in as many as six phases,² but it is generally subdivided into three main functional stages universally referred to as the presecretory, secretory and maturation stages of amelogenesis.³ Each stage can be divided into regions based on characteristic morphological features of ameloblasts and/or the appearance of the extracellular matrix they produce.⁴ These regions can themselves be subdivided into smaller functional units as, for example, the region of ameloblasts facing dentin which can be partitioned into three subregions.⁵ Classically, ameloblasts from each stage are portrayed as fulfilling more or less exclusive functions: first, during the *presecretory stage* differentiating ameloblasts acquire their phenotype, change polarity, develop an extensive protein synthetic apparatus, and prepare to secrete the organic matrix of enamel; second, during the *secretory stage* ameloblasts elaborate the entire enamel thickness by appositional growth and simultaneously organize it into characteristic rod/interrod regions; and third, during the *maturation stage* the ameloblasts modulate and transport specific ions required for the concurrent accretion of mineral.^{2,6} These combined activities result in the formation of a highly ordered tissue containing over 96% mineral by weight, the hardest in the body. Over the past ten years or so, the cellular activities occurring during each stage have been more extensively investigated, and ameloblasts must now be viewed as cells which carry out multiple activities throughout their life cycle and which upregulate, or downregulate some or all of them accordingly.

II. MORPHOLOGICAL EVENTS OF AMELOGENESIS

The compartmentalization of the ameloblast life cycle into stages implies that enamel development occurs in precisely defined steps and that extracellular matrix proteins are produced and released exclusively at one point in time — the secretory stage. Early ultrastructural studies described the exist-

ence of a granular material of varying texture associated with presecretory stage ameloblasts. This material is visible even before there is any disruption in the basement membrane that separates differentiating ameloblasts from odontoblasts and the developing dentin.⁷ Such granular material, also referred to as "stippled material",⁸ is believed to represent a form of precursor enamel proteins (EPs).^{8,9} Radioautographic studies have shown that presecretory stage ameloblasts incorporate radiolabeled amino acids and synthesize and secrete proteins in a manner similar to secretory stage ameloblasts.¹⁰⁻¹³ Such studies have also indicated that the granular material seen between collagen fibrils in the forming mantle dentin is derived from ameloblasts.¹⁰ In the rat incisor, the first EPs are immunodetected within the protein synthetic and secretory organelles of ameloblasts soon after they have completed their last mitotic division and are only just beginning to undergo differentiation.¹² EPs are also found in lysosomal elements, particularly multivesicular bodies,^{12,14,15} which are abundant in late presecretory stage ameloblasts.¹⁶ In humans, EPs have also been observed in coated pits at the apical surface of these cells.¹⁴ Extracellularly, EPs are first immunodetected in association with the *lamina fibroreticularis* of the basement membrane separating the differentiating ameloblasts and odontoblasts, as early as when matrix vesicles are still intact in the developing mantle predentin (Figure 1).^{12,14,15,17} Fibronectin is present in the basement membrane at this time,¹⁸ and the EPs may be involved in the spatial redistribution of this molecule that could affect final odontoblast differentiation and polarization.¹⁸ The EPs then accumulate as patches of granular matrix between collagen fibrils within the forming mantle dentin (Figure 2). These patches eventually give rise to, or are replaced by, a continuous layer of initial enamel (Figures 2 and 3). During this sequence of formation, three proteins are believed to be expressed sequentially: the first two appear to be anionic higher molecular weight EPs (nonamelogenins), followed by the more classic amelogenins.¹⁷ Since EPs first appear in forming mantle predentin prior to the onset of mineralization in this extracellular matrix,^{17,19} these proteins may participate in the initial events of tooth mineralization. Material with EP-like antigenicity and texture is also seen deep within the forming mantle dentin and along odontoblastic processes (Figure 3), as well as between the odontoblasts themselves.^{12,14,15,20} EPs have been immunolocalized within coated elements and multivesicular bodies of young odontoblasts facing differentiating ameloblasts in rodent molars,²⁰ but not in rodent incisors. In addition, EPs are present along odontoblast processes throughout amelogenesis and not just at the early stage in rat incisors (Figure 4).¹⁹ These data raise the possibility that EPs may diffuse passively from developing enamel into the adjacent dentin throughout amelogenesis.

The bulk of the enamel matrix is produced during the secretory stage. Both radioautographic and immunocytochemical studies¹² have shown that EPs follow the classical protein secretory pathway.²¹ As the proteins move from the rough endoplasmic reticulum through the Golgi apparatus, they are

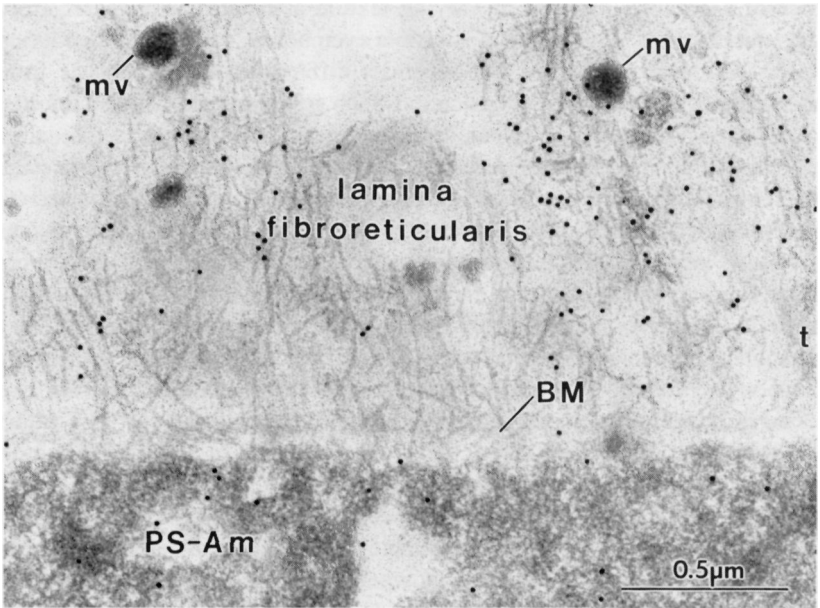


FIGURE 1. Rat incisor: EPs (immunodetected with mouse anti-amelogenin antibody [courtesy of H.C. Slavkin] and revealed by protein A-gold) are seen very early within the extracellular matrix during differentiation of presecretory stage ameloblasts (PS-Am) and initially accumulate within the *lamina fibroreticularis* of the apical basement membrane (BM); mv, matrix vesicle.

posttranslationally modified and packaged into immature secretory granules which bud from the trans Golgi network. The proteins then condense to form mature secretory granules. The mature granules move rapidly toward the Tomes' process under the guidance of microtubules,²² where they accumulate briefly. The granules ultimately release their contents at two primary sites characterized by extensive membrane infoldings.^{23,24} The interrod secretory site is situated circumferentially around the proximal portion of Tomes' process in proximity to the interrod growth site.²³ The rod secretory site is found only along one surface of the distal portion of Tomes' process and is in contact with the growing end of the rod.^{23,24} Exocytosis occurs by a merocrine-like mechanism, whereby the granules fuse either directly to the surface membrane²⁵⁻²⁷ or to deep membrane infoldings.²⁸ Considerable membrane recycling likely occurs in ameloblasts, and the large amount of cell membrane devoted to these infoldings suggests that they may arise because there is rapid fusion of massive numbers of secretory granules at these sites. It is also possible that secretory granules fuse indirectly to the cell membrane by means of tubular channels interconnecting the granules.^{29,30} This could provide a mechanism for simultaneous exocytosis of the content of clusters of secretory granules.²⁹ Although numerous coated pits and coated vesicles are observed

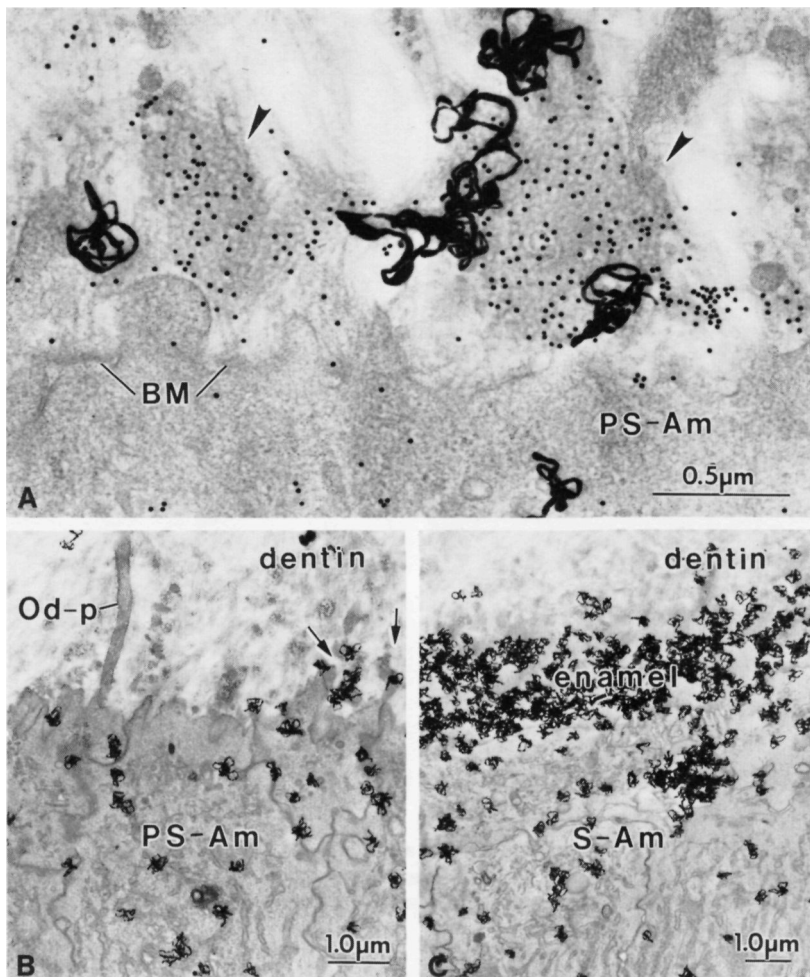


FIGURE 2. Rat incisor: patches of newly formed immunoreactive (mouse anti-amelogenin) granular material (arrowheads) start accumulating at the apices of presecretory stage ameloblasts (PS-Am) by 20 min after injection of ^3H -methionine (A). At 1 hour after injection, few radiolabeled products are seen at the apices of presecretory stage ameloblasts (B), while secretory stage ameloblasts (S-Am) show the beginnings of appositional growth of the enamel layer (C); Od-p, odontoblast process.

in the vicinity of the secretory sites, no EPs have been immunolocalized within them during the secretory stage in the rat incisor.^{15,11} Since EPs are seen in coated vesicles of odontoblasts,²⁰ the apparent lack of immunolabeling over these structures in ameloblasts suggests that either (1) the ameloblasts pick up few EPs at the apical surface; (2) the proteins they endocytose are not recognized by antibodies to EPs; (3) the EPs move very quickly through

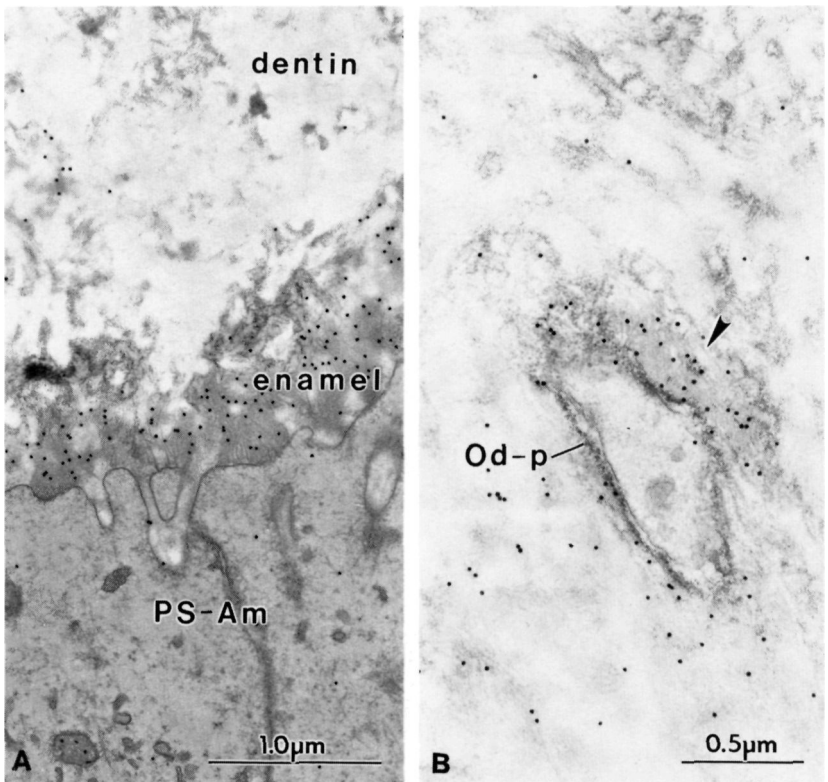


FIGURE 3. Cat: EPs (immunodetected with antibody to pig LRAP proteins [courtesy of H. Limeback]) at the apices of presecretory stage ameloblasts (PS-Am) gradually form a continuous layer of enamel. During this stage immunolabeled products are found along the odontoblast processes (Od-p).

this intracellular compartment; or (4) the ameloblasts use some structure other than a coated vesicle as an intermediate to transfer resorbed EPs to the lysosomal compartment.³² There are smooth tubular structures in Tomes' process that often show weak immunolabeling for EPs and may correspond to an early endosomal compartment in this region of the cell.³³

Morphologically, two types of mature secretory granules are recognizable in Tomes' process. In calcified specimens, these clearly appear as granules with a pale or dark content. Following incorporation of ³H-methionine, only a portion of the granules are labeled, a finding which is consistent with fast turnover for these granules. Combined immunocytochemical and lectin cytochemical studies reveal three populations of granules: granules reactive to mouse anti-amelogenin only, granules reactive to wheat germ agglutinin only, and granules reactive to both probes.³⁴ Secretory granules are also variably reactive by acid phosphatase cytochemistry.^{29,30} One major question is whether

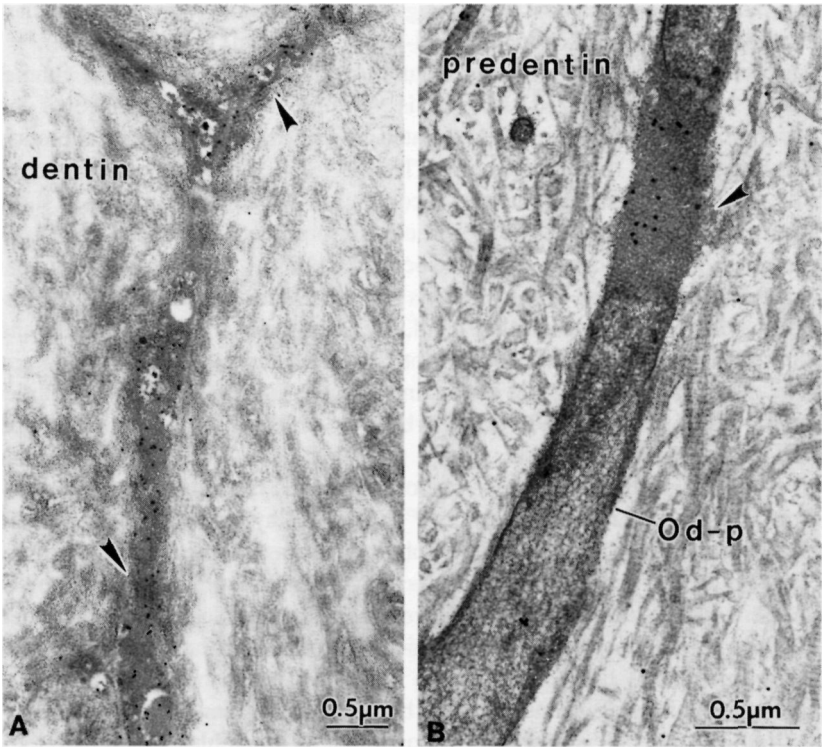


FIGURE 4. Rat incisor: material reactive to mouse amelogenin antibodies is found in dentin (A) and along odontoblast processes in pre-dentin (B; Od-p) even during the maturation stage.

the two main classes of EPs (enamelin and amelogenin) are packaged within the same, or separate, secretory granules. Such an issue has been especially difficult to resolve because antibodies raised to what is believed to be distinct classes of EPs often cross react between what appears to be nascent amelogenins and enamelin or their immediate degradative products.³⁵ However, a recent double-labeling immunocytochemical study with monospecific polyclonal antibodies to bovine amelogenins and enamelin indicates that both proteins often colocalize within the same secretory granule.¹⁴

Maturation stage ameloblasts have been viewed as cells that carry out major resorptive and transport functions and little biosynthetic activity.³⁶ The most visually dramatic activity of these cells is modulation, the cyclic creation, loss, and recreation of a highly invaginated ruffle-ended apical surface.³⁷ Available evidence suggests that ameloblasts in some species modulate extremely rapidly, as often as once every 8 hours, thereby yielding 3 complete modulations per day.³⁸ The modulations can be visualized by special stains^{39,40} and occur in waves traveling across the crown of a developing tooth from

least mature regions to most mature regions of the enamel (e.g., apical-incisal direction in rodent incisors or cervical-incisal [occlusal] in bovine teeth).^{38,41} Ameloblasts modulate during the maturation stage, at least until the enamel becomes fully mineralized. No one is yet sure of the significance of the modulations, but they seem to be related to calcium transport and alteration of permeability in the enamel organ. That is, ruffle-ended ameloblasts typically are tightly bound by junctional complexes at their apices.³⁷ They show considerable endocytotic activity and contain numerous lysosomes, calcium-binding proteins, and membrane-associated calcium ATPases which appear to promote the pumping of calcium ions into the maturing enamel.⁴²⁻⁴⁴ Smooth-ended ameloblasts, on the other hand, are very leaky to small proteins and other molecules, show little endocytotic activity, and almost no membrane calcium ATPase activity.⁴²⁻⁴⁴ Interestingly, there is a point in time, as ameloblasts recreate the ruffled border, where the cells are so tightly bound and deficient in calcium ATPase activity, that practically no calcium ions enter the enamel for a brief period.⁴² Ameloblasts spend the majority of the modulation cycle in the ruffle-ended state.³⁷ It has been hypothesized that repeated modulations to the smooth-ended morphology are necessary in order to release tissue fluids into the enamel layer to neutralize the low pH caused by growth of the enamel crystals.³⁸ Direct evidence supporting this idea has been obtained recently in bovine incisors, where the microenvironment extracellular to smooth-ended ameloblasts has been found to be more neutral in pH, compared to more acid regions of enamel covered by ruffle-ended ameloblasts.⁴⁰

Radioautographic studies have shown that as ameloblasts modulate they also incorporate radiolabeled precursors and secrete proteins, some of which may be components of the inner basal lamina reformed at the apices of maturation stage ameloblasts.^{12,45} Recent biochemical, immunocytochemical, and radioautographic studies have shown that early maturation stage ameloblasts still produce a variety of cellular and structural proteins, as well as EPs, some of which are secreted by the ruffle-ended ameloblasts.^{12,13} The exact biochemical nature of all proteins secreted during the maturation stage, as well as the reason for continued addition of new EPs at the enamel surface while the majority of older ones are being degraded and removed, remains to be elucidated.

III. PROTEINS SECRETED BY AMELOBLASTS INTO DEVELOPING ENAMEL

It was evident from early biochemical studies that proteins found within developing enamel differed biochemically from those present in other hard tissues, such as bone and dentin, in which loss of major quantities of organic material does not occur as part of the mineralization process.³⁵ The high sulfur content and insoluble nature of proteins extracted from young enamel, but not from mature enamel, led some earlier researchers to suspect that the

transient component in enamel might be some type of keratin protein.^{46,47} While this issue was never completely resolved,^{48,49} numerous biochemical experiments done over the past 30 years have established that at least two distinct types of matrix proteins may be deposited in developing enamel.^{35,46,50,51} These can be distinguished crudely on the basis of molecular weight, amino acid composition, and solubility, and, to a lesser extent, by antigenicity. The two types are (1) the relatively small (22 to 30 kDa by SDS-PAGE), proline-rich, hydrophobic amelogenins, which are prone to aggregate;^{35,46,50-54} and (2) the relatively large (48 to 70 kDa by SDS-PAGE), glycine-rich, hydrophilic amelins, which are acidic and likely carry sugar and phosphate groups on certain amino acids.^{35,46,51,55-57} It is broadly accepted that the amelogenins comprise the largest amount (>90%) of total proteins released by ameloblasts during the secretory stage.^{35,51} There is also general agreement that proteins of the amelogenin group constitute the main organic component of developing enamel, which disappears during the maturation stage when the total mineral content of this tissue increases.^{35,46,50,51,54}

Practically every other aspect of the biochemical, immunochemical, genetic, and physical properties of both classes of EPs, but especially the amelins, remains extremely controversial. In the case of amelogenins, the genes coding for this protein have been localized to the X- (bovine, mouse, human) and Y-chromosome (bovine, human),^{58,60} but there is considerable debate as to whether ameloblasts translate one protein, or as many as four separate amelogenins, at the level of the rough endoplasmic reticulum.^{35,59,61} It is also unclear what posttranslational modifications this protein receives along the secretory pathway.^{35,52,54} There is evidence that a leader sequence of about 19 amino acids is cleaved from amelogenin somewhere in the interval between when the protein is released into the lumen of the rough endoplasmic reticulum and when it is exocytosed from a secretory granule.⁶² There has been considerable debate whether ameloblasts initially manufacture a "high molecular weight" proamelogenin (e.g., 58 kDa), which is then cleaved into multiple proteins (e.g., 28 and 30 kDa), either just before or just after the proamelogenin is secreted by ameloblasts.³⁵ Other workers have argued that "high molecular weight" amelogenins detected in enamel are aggregate forms of nascent low molecular weight amelogenins.^{35,53} There has been good agreement about the primary sequence of amino acids for the "major" amelogenin in various species (bovine, pig, mouse, human) but noticeable disagreement as to the exact molecular weight of this protein between species.³⁵ In the case of amelins, the very existence and nature of these proteins have been argued ever since Termine and co-workers published a protocol for their isolation by differential extraction in 1980.⁵¹ In their original description, amelins were conceptualized as long-lived, acidic, and phosphorylated glycoproteins of about 70 kDa, which played a role either in "seeding" mineralization or in controlling crystallite growth by acting as a covering at the surface of hydroxyapatite crystallites, or both.^{35,46,51} It was suspected that partially

degraded enamelin remains associated with crystals in mature enamel while amelogenins, whose job was to fill the spaces between growing crystallites, would break down and/or disaggregate and be removed completely from maturing enamel.^{35,46,51} There has been some controversy as to whether enamelin undergoes partial degradation during the secretory stage and/or early maturation stage of amelogenesis, or only during late maturation.³⁵ It is also unclear if enamelin degrades only to a certain level and no further.⁵⁵ Some workers suspect that degradation products from enamelin may have a molecular weight that is very similar to "primary" amelogenins and/or degradation products of amelogenins.^{35,53,57} However, it is evident from recent literature that different workers have reported different proteins as "enamelin".⁶³ It is also evident that some of the "enamelin-like" proteins that have been purified and described are really serum proteins such as albumin, a2HS-glycoprotein, and g-globulin, or their degradation products.⁶⁴⁻⁶⁶

In addition to amelogenins and enamelin, there is increasing evidence that ameloblasts release enzymes, especially endoproteinases, into developing enamel.⁶⁷⁻⁷⁰ The literature on enamel proteolytic enzymes, like the enamel matrix proteins, has been controversial, with little agreement between various investigators as to the exact class(es) of enzymes present in developing enamel.⁶⁷⁻⁷⁰ There has also been an obvious uneasiness concerning the reality of such enamel-resident extracellular enzymes. That is, cells which cover developing enamel are full of proteolytic enzymes, and the cells must be removed in order to obtain pieces of enamel for biochemical analysis. The chances of contaminating the enamel with soluble cellular proteins is clearly very high, even under freeze-dried conditions where not all cellular debris can be removed with absolute certainty. Nevertheless, enough evidence has accumulated to suggest that enamel proteinases are real and that these enzymes are present in enamel in an active form continuously from the secretory stage through the midmaturation stage of amelogenesis.^{67,69-71} At the moment, there appear to be several metalloproteinases and at least one serine proteinase in developing enamel.^{67-70,72} Of interest have been reports of a unique serine proteinase that is active during the maturation stage and which may play a role in specifically degrading amelogenins.⁶⁷⁻⁷⁰ It is suspected that the actions of enamel proteinases may be organized as a cascade, such that different enzymes act on different parts of the amelogenin and/or enamelin protein.^{67,69,70} It is also suspected that one level of degradation may lead to another lower level of sequential degradation over time.^{67,70,72} There is evidence that one of the metalloproteinases in enamel is stromelysin (matrix metalloproteinase-3) and/or type IV collagenase.^{67,72,73} Extracellular proteinases are invariably also associated with natural activators and inhibitors, but these have not yet been identified with any certainty in enamel.^{67-70,72-75} Similarly, it is not clear whether the proteinases need to be replenished regularly (turnover), or what is their ultimate fate once the majority of EPs (amelogenins) have been removed during maturation. Besides proteinases, there have

also been reports of certain other enzymes in enamel, such as alkaline phosphatase.^{71,76} The role of such enzymes in enamel development is undefined at present.

There may be another traditional extracellular matrix component in enamel, that is, glycosaminoglycans (proteoglycans) and/or sulfated glycoproteins.⁷⁷⁻⁸⁰ Extracellular soft and hard tissues generally contain proteoglycans,⁸¹ and it has been conceptually difficult to understand their absence in enamel, making it the only hard tissue lacking such an extracellular component.⁸¹⁻⁸⁶ This is especially important in light of several reports of a key role that proteoglycans may play in controlling mineralization of cartilage,⁸⁴ bone,⁸² and dentin.⁸³ Related to this issue is an observation that has always been puzzling, that is, the high sulfur content reported for newly formed enamel.^{47,87,89} Amino acid compositions for EPs have indicated less than 6% of methionine in amelogenins and less than 2% of methionine in enamelines, with neither apparently containing much cysteine.^{35,46,50,52,55,57,63} Since the sulfur in developing enamel cannot be ascribed to amino acids of constituent proteins, it must therefore be derived from sulfate. Sulfate is a common component of proteoglycans and can also be attached to the sugar groups of glycoproteins or even directly to tyrosine in proteins.^{81,90-93} Several workers have reported considerable uptake of sulfate by secretory stage ameloblasts and eventual transfer to some ill-defined molecule secreted into the enamel layer.^{47,87,88} Of interest have been reports that sulfate labeling is lost relatively rapidly from secretory stage enamel and eventually disappears completely in maturation stage enamel.^{44,47,88}

Lastly, while the precursors of ameloblasts — the inner enamel epithelium — are largely responsible for producing the basement membrane which separates the inner surface of the enamel organ from the pulp and developing mantle dentin, the ameloblasts are responsible for removing this basement membrane prior to the start of the secretory stage,⁹⁴ as well as for producing the components of the inner basal lamina when it is later reapplied to the enamel surface at the start of the maturation stage of amelogenesis.^{45,95} The identification of matrix metalloproteinases in developing enamel^{62,72,73} suggests that ameloblasts never completely suppress production of some of their basement membrane components and may continue to secrete small quantities of proteins, such as type IV collagen, that intermix with the much larger quantities of enamel-specific proteins. These components may simply degrade too quickly, or exist in too small quantities, to be detected biochemically during the secretory stage of amelogenesis.

IV. REMOVAL OF PROTEINS FROM DEVELOPING ENAMEL

Besides the concepts of dual categories of EPs (enamelines and amelogenins), and of extracellular proteinases, another idea which has had

significant impact on enamel research has been the hypothesis that ameloblasts resorb intact or partially degraded EPs directly from maturing enamel and dispose of them within their lysosomes.⁸⁸ At the time it was proposed, and for the following 20 years, this concept provided a reasonable explanation for why ameloblasts possess such a complicated lysosomal system and why maturation stage ameloblasts, especially those which are ruffled-ended, frequently show numerous large acid phosphatase- and trimetaphosphatase-positive lysosomes.^{29,30,44,45,96-100} Studies with exogenous protein tracers have clearly demonstrated that ruffle-ended ameloblasts avidly endocytose intravascularly injected tracers and transfer them rapidly to their lysosomes.^{42,44,101,102} Endocytotic activity in ruffle-ended ameloblasts seems greater than in smooth-ended ameloblasts, or compared to secretory stage ameloblasts.¹⁰¹⁻¹⁰³ Recent immunocytochemical studies, using different monoclonal and polyclonal antibodies to either amelogenins or enamelin, have consistently demonstrated the presence of immunoreactive material within lysosomes of ameloblasts.^{14,15,31,104} While highly suggestive of resorptive activity, this evidence, as well as that obtained by cytochemical techniques (tracers), prove only that ameloblasts display active fluid-phase endocytosis and that there are EPs, or their immediate degradative products, within the lysosomes of ameloblasts.

Enzyme inhibitor studies have revealed that extracellular degradation of EPs can be blocked using natural compounds such as aprotinin, an inhibitor for trypsin-like serine proteinases.⁷⁰ It is also possible to block intracellular degradation of EPs within lysosomes of ameloblasts using other natural compounds such as leupeptin, an inhibitor for thiol proteinases (cathepsins B/H/L), and serine proteinases.^{13,70} Immunocytochemical studies have indicated that the lysosomes of leupeptin-treated ameloblasts are filled with immunoreactive material as monitored by an anti-amelogenin antibody.¹⁰⁵ Radioautographic studies have further shown that much of the excess material accumulating within these lysosomes corresponds to newly formed proteins.^{13,105} In contrast, there is no solid evidence from radioautographic studies for a natural increase in lysosomal labeling in ruffle-ended ameloblasts at 4 to 8 days after a single pulse injection of ³H-methionine, ³H-leucine, or ³H-glycine, when aged, radioactive EPs are lost from maturing enamel.^{13,70,104,106} These results suggest that newly formed material passing into the lysosomal system of ameloblasts is degraded rapidly and the byproducts are likely not conserved but are returned to the general circulation. It should be noted that the anti-amelogenin antibodies used in many published studies cannot reveal proteins less than 14 kDa in molecular weight as determined by immunoblotting.¹²¹³ Consequently, it cannot be stated with absolute certainty that EPs degraded to this size or below do not gain access to the lysosomes of ameloblasts at some point in time. Existing radioautographic data suggest that this does not occur but such evidence remains largely circumstantial.^{104,106}

VECTORIAL SECRETION

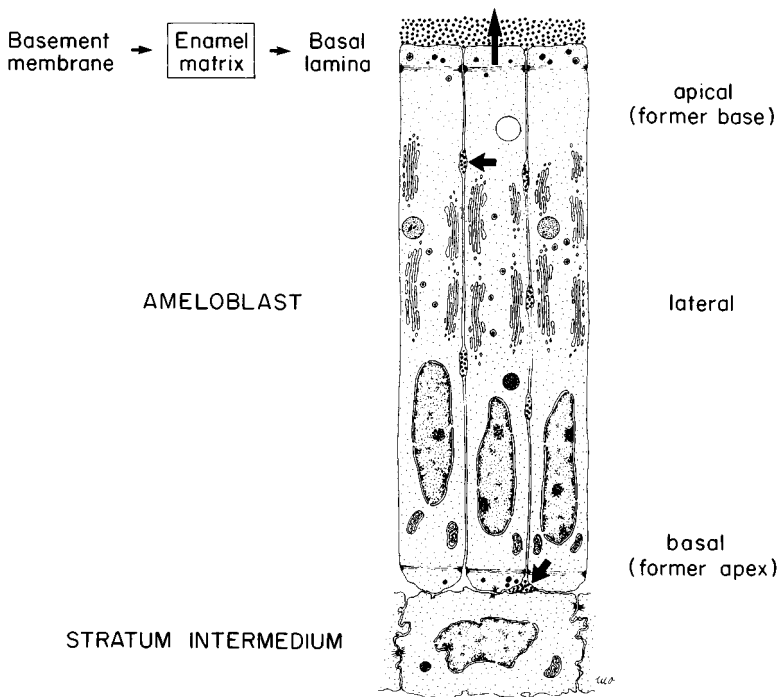


FIGURE 5. Schematic drawing illustrating the concept of vectorial secretion in ameloblasts. While most secretory products go to the functional apex (large arrow), a portion may get routed basolaterally (short arrows) as a consequence of the reverse polarity this cell undergoes during differentiation.

There is another possible explanation for the origin of immunoreactive EPs detected in lysosomes of ameloblasts. That is, ameloblasts may secrete as much as 10% of the proteins they biosynthesize to their lateral and basal surfaces, and then quickly reincorporate this material back into lysosomes by fluid-phase endocytosis. It is well documented that polarized epithelial cells direct some proteins exclusively to their apical surfaces, while others are directed to the basal and lateral surfaces.¹⁰⁷⁻¹¹¹ A special case could exist in ameloblasts because of the reverse polarity they undergo during differentiation (few epithelial cells do this; Figures 5 and 6). Before inner enamel epithelial cells become preameloblasts, the Golgi apparatus is located on the side of the nucleus situated toward the stratum intermedium. As preameloblasts differentiate, the Golgi apparatus moves to the opposite pole of the nucleus toward the basement membrane with preodontoblasts. Consequently, what constituted the base of the cell becomes the functional apex, and it is along

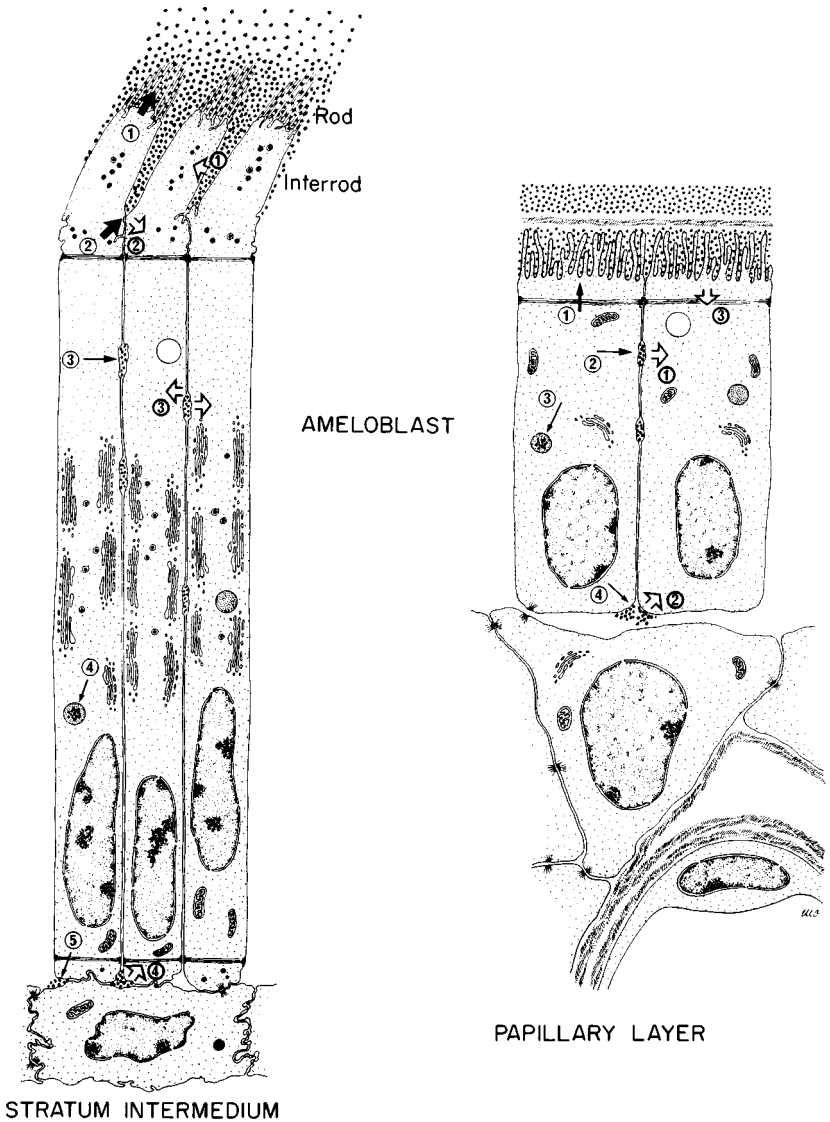


FIGURE 6. Schematic drawing illustrating possible directions of flow of proteins out of the secretory pathway (numbers with thin circles) or into the lysosomal pathway (numbers with thick circles) of ameloblasts. Secretory stage: *Out*, 1 = rod; 2 = interrod; 3 = lateral; 4 = lysosomal; 5 = basal; *In*, 1 = distal portion Tome's process; 2 = proximal portion Tome's process; 3 = lateral; 4 = basal. Maturation stage: *Out*, 1 = apical; 2 = lateral; 3 = lysosomal; 4 = basal; *In*, 1 = lateral; 2 = basal; 3 = apical.

this surface where the bulk of the EPs (and later proteinases) are ultimately secreted (Figures 5 and 6).^{12,29,31,35,104,106} This restructuring may upset the normal targeting mechanisms which direct appropriate "apical proteins" to "apical surfaces" (Figures 5 and 6). Secretory granules are sometimes seen in clusters in the functional base of ameloblasts (most prominent during the secretory stage), and it is possible that some or all of these granules may be exocytosed and replaced by new granules that are also exocytosed. Pools of recently formed immunoreactive material are often seen naturally in the basolateral spaces between ameloblasts (Figures 7 to 9),^{14,112} and this could be derived from the contents of such basolaterally exocytosed granules. An extreme example of basolateral secretion by ameloblasts is seen in animals treated with compounds that disrupt microtubules and filaments (Figure 9). These ameloblasts often show large amounts of immunoreactive, granular material surrounding the sides and base of the cell (Figure 9).^{113,119} This presumably happens because secretory granules that would normally go to the apical surface (90% of secretory products) become "disoriented" and release their contents at the first plasma membrane encountered. This, in fact, would most often be the lateral sides of the cell, since the Golgi apparatus becomes fragmented and dispersed throughout the cytoplasm of the ameloblast affected by these compounds.¹¹⁵⁻¹¹⁹

The possibility that some EPs in lysosomes also derive from endocytosis at the apical surface and/or from direct targeting of secretory proteins to lysosomes cannot be ruled out completely (Figure 6). Indeed, the 10% of EPs suspected to be cycling through the lysosomal system is consistent with quantities of exportable proteins degraded in other active secretory cell types, such as fibroblasts.¹²⁰ The predominance of multivesicular bodies in the Golgi region of ameloblasts (Figure 6), and their relative paucity at sites close to where endocytosis may occur (e.g., basal surface), are not inconsistent with the above theory, considering recent evidence for funnelling of apical and basolateral endocytotic pathways to a common lysosomal compartment associated with the apical region of polarized cells.^{108,121} It must also be recognized that several distinct types of multivesicular bodies can be identified in ameloblasts and some may be related to endocytosis, while others may be involved in posttranslational degradation.¹²²⁻¹²⁴ Pale multivesicular bodies often represent an intermediate in the evolution of endosome to lysosome^{32,121,123,125} and their relatively weak immunolabeling¹⁰⁴ may reflect endocytosis of extracellularly processed proteins which are no longer recognizable by immunocytochemical methods, at least with the antibodies we have used.

Lastly, it is important to recall that the original concept of resorptive activity by ameloblasts was proposed many years ago, before it was realized that ameloblasts may secrete extracellular proteolytic enzymes,^{67-73,106} and before it was widely accepted that amelogenins likely undergo considerable degradation by these enzymes in a "top-down" fashion from some precursor (e.g., 25 kDa by SDS-PAGE) toward a much lower molecular weight

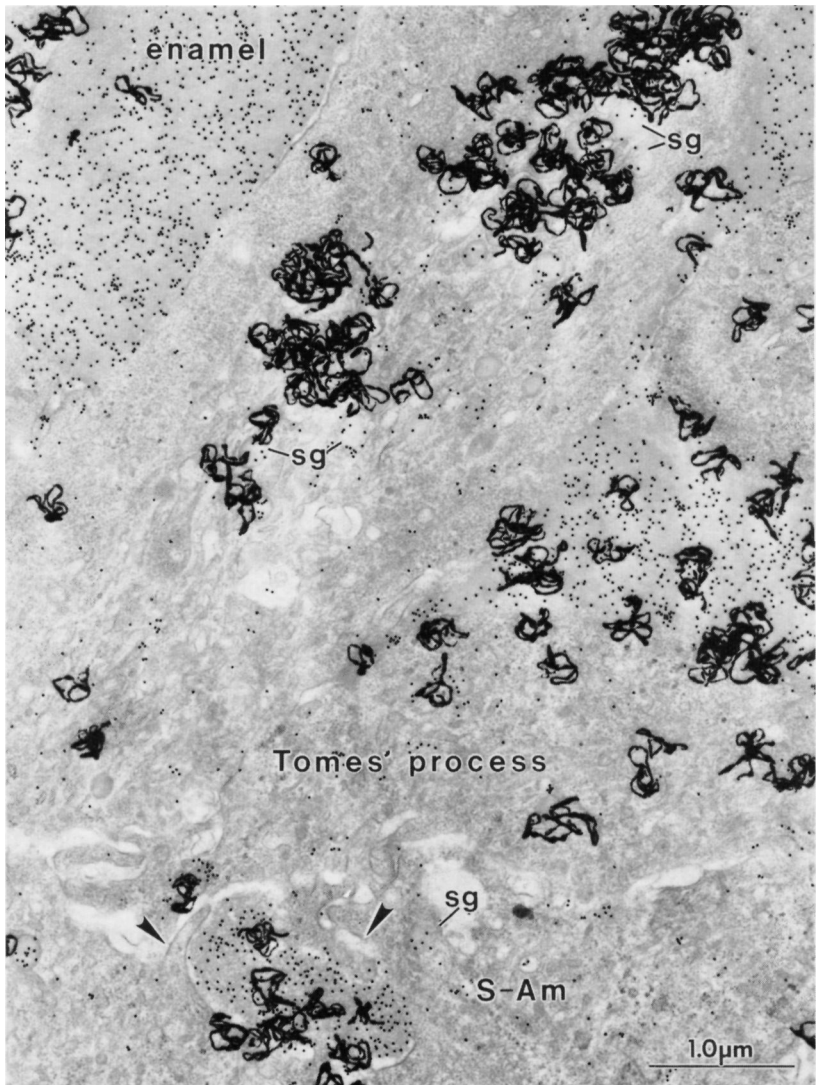


FIGURE 7. Rat incisor: as shown by combined protein A-gold immunocytochemistry and radioautography, some recently formed products (arrowheads) are released at the lateral surfaces of secretory stage ameloblasts (S-Am); sg, secretory granules.

byproduct (e.g., 6 to 10 kDa by SDS-PAGE).^{35,46,50,54,106} While some researchers argue that amelogenins are naturally in an aggregated state and become disaggregated during maturation,⁵³ the "top-down" proteolytic processing must still occur, as suggested by the findings of *in vivo* pulse-labeling studies done in rodents,^{106,126-128} which show *in situ* lowering of the molecular

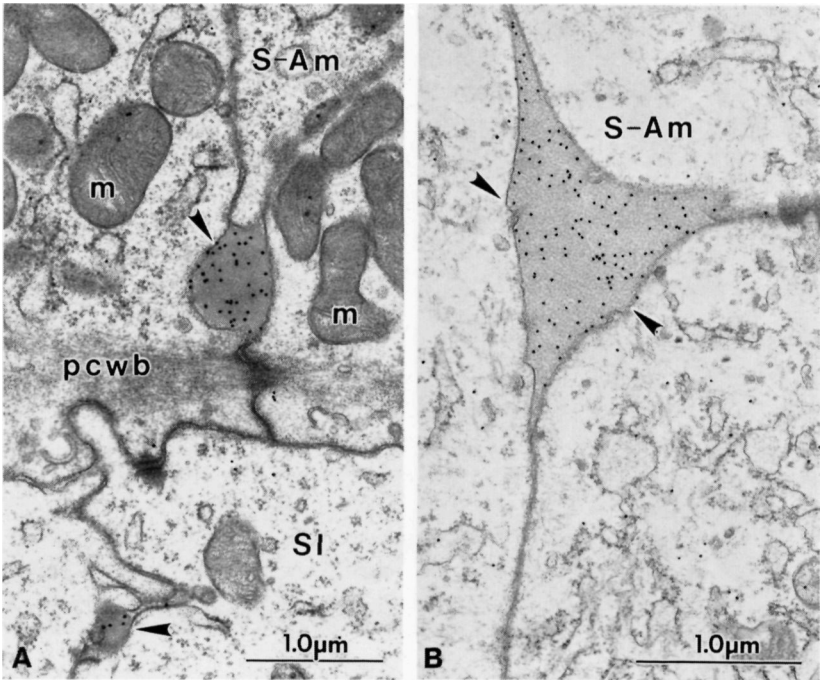


FIGURE 8. In both mouse (A) and cat (B), immunoreactive material (arrowheads) is observed at the basolateral surfaces of secretory stage ameloblasts (S-Am). m, mitochondria; pcwb, proximal cell web; SI, stratum intermedium.

weight of EPs as they age (mature). Furthermore, *in vitro* studies have indicated that pieces of developing enamel can be removed and incubated without associated cells in a moist environment and the constituent matrix proteins will undergo substantial proteolytic breakdown.^{67,70,106} This *in vitro* breakdown can be blocked by preboiling the samples (which kills the enzymes) or by incubating the sample in the presence of certain inhibitors, such as PMSF or SDS.^{67,70} It is characteristic of *in vitro* incubations that the EPs present in the original sample become unresolvable by electrophoresis; that is, they are degraded to a molecular weight low enough to run off the bottom of the polyacrylamide gels.^{67,70} Other studies have shown that the degree of *in vitro* protein degradation varies according to the source of the sample.^{70,106} Proteins in secretory stage enamel break down slower and to a lesser extent than proteins in early maturation stage enamel, while proteins in midmaturation stage enamel break down completely within 2 days.^{70,106} Cross stage experiments have indicated that secretory stage EPs, for example, will break down completely within 2 days if mixed with enzyme extracts taken from midmaturation stage enamel.⁷⁰ Hence, it is the potency or amount of enzymes in the developing enamel that dictates how rapidly the matrix proteins will

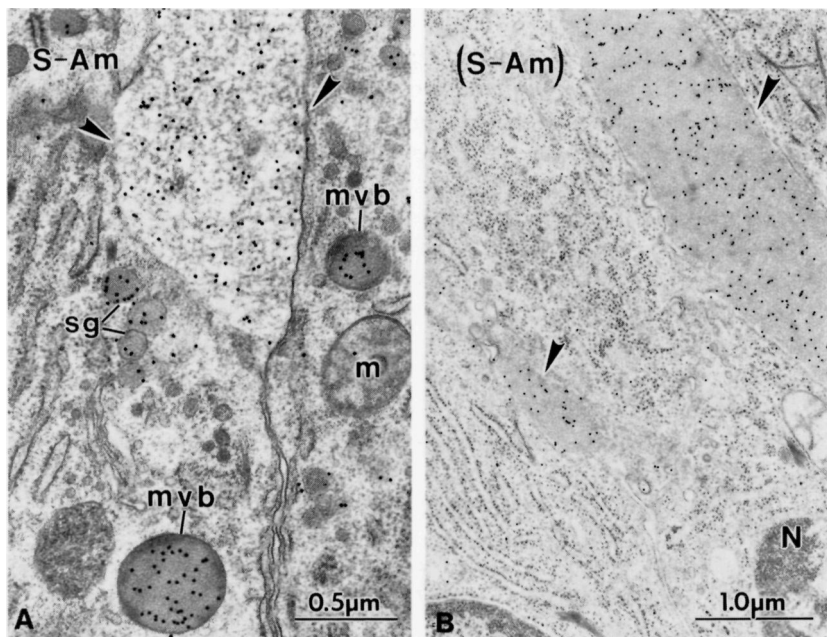


FIGURE 9. The basolateral release of immunoreactive products (arrowheads) can be accentuated either following administration of vinblastine (A, rat incisor), or by growing tooth germs in organ culture (B, mouse molar culture [courtesy of H.C. Slavkin]). m, mitochondria; mvb, multivesicular body; N, nucleus; S-Am, secretory stage ameloblasts; sg, secretory granules.

break down. Recent kinetic experiments with fluorescing substrates have indicated that the enzymes themselves have variable stability on long-term incubation *in vitro* (unpublished). Some enzyme activities are lost within a few hours, while others remain stable for many days. This suggests that ameloblasts must continuously add some new enzymes to the developing enamel for those activities which are unstable and deposit only once other enzymes which are long lived.

From the above discussion, we propose that ameloblasts likely control protein loss in maturing enamel by means of the enzymes they secrete. That is, it seems more reasonable that these cells would attempt to control the activity of a few picograms of enzyme and what they degrade (both specifically and nonspecifically), rather than try to control the bulk movement of several hundred micrograms of protein, as would be necessary if the apical surfaces of ruffle-ended ameloblasts were the exit point for proteins. The problem of attempting to control the loss of protein by purely physical means (i.e., resorption) becomes even more unlikely, given that two distinct categories of proteins (enamelin and amelogenin), with differing solubilities (hydrophilic vs. hydrophobic) and life span (stable vs. transient) may exist in developing enamel. The purpose of the enzymes would likely therefore be to

reduce the molecular size of EPs to a level where they would become freely soluble within the fluids that surround growing crystals (the so-called enamel fluid).¹²⁹ These solubilized fragments could then diffuse randomly out of the enamel layer when ameloblasts are in the smooth-ended phase and presumably leaky at their apical ends.^{129,130} We also suspect that enamelin and amelogenin are equally degraded to a point that they dissolve in the enamel fluid and leave the system. As mentioned earlier, it remains equivocal whether ameloblasts ultimately endocytose (resorb) some of the water-soluble fragments and dispose of them in lysosomes. If they do so, it may be done from the lateral surfaces of the cell, as the ruffled borders are being recreated on initiation of each new modulation cycle.^{38,45,129,131,132}

V. FORMATION AND GROWTH OF ENAMEL CRYSTALS

The cells controlling formation and mineralization of enamel are epithelial in nature. Consequently, the chemical and physical properties of the proteins secreted by ameloblasts of the enamel organ, and their physical association to the surface of the mineralizing extracellular layer, are different from relationships typically seen for formative cells in the collagen-based hard tissues (mesenchymal origin). There has been considerable argument about whether ameloblasts make components required to initiate crystals of hydroxyapatite.^{133,134} This is because most of the extracellular matrix proteins associated with the developing enamel crystals are predominately hydrophobic and globular in nature, and they are degraded and lost over the course of development.^{35,50,54,106} The apical plasma membranes of the ameloblast are positioned near the sites where crystals of hydroxyapatite are being produced, but unlike other hard tissues, the plasma membranes of ameloblasts are pressed tightly against crystals that are growing in length. Later in time, these cells are located several hundred to several thousand micrometers away from the same crystals when they undergo secondary growth in thickness and width during the maturation stage of amelogenesis.³ There is also no obvious mineralization front in enamel demarcating a region in space where a purely organic precursor layer is transformed to a hard tissue by mineralization, and EPs therefore seem to participate in mineralization events the instant they are released extracellularly.^{1,135} Furthermore, the complex three-dimensional organization of enamel appears to be based more on direct ordering of the crystals of hydroxyapatite (inorganic component) than on ordering of a structural matrix protein (organic component), as is seen, for example, with the collagen fibrils in lamellar bone.¹³³

The fundamental organizational units in mature mammalian enamel are the rods (prisms) and the interrod material (interprismatic substance).^{1,135} Both are built from thin, closely packed, and extremely long, ribbon-like crystals of hydroxyapatite.^{136,137} The interrod enamel appears first and forms an array

of cavities which are progressively lengthened and filled in by the rods.¹³⁸ In most species, rods appear to bend and twist along irregular paths. Rods are surrounded on all sides by interrod enamel, which begins as a thin aprismatic layer applied against the dentino-enamel junction (initial enamel or inner prismless layer) and extends as a thin covering over the ends of the rods at the surface (final enamel or outer prismless layer).^{1,135} The crystals of rod and interrod enamel are similar in structure but differ in their relative orientation.¹³³ In the case of rods, the crystals are believed to be organized by the interdigitating portions of Tomes' processes, and arranged so that the general direction of their c-axes run parallel to the long axes of the rod, while their a- and b-axes may be at any angle.^{23,133} The Tomes' process of one ameloblast is believed to organize one enamel rod.^{1,139} Interrod enamel is formed in relation to the proximal portions of Tomes' processes and its deposition and organization, around a single rod, represents the cooperative effort of several cells.²³ Its crystals have their c-axes positioned at any angle other than parallel to the long axes of the rods.¹³³ Crystals traverse the enamel thickness and follow a very tortuous path. It has been suggested that groups of crystals, particularly in rods, possess an overall helical organization.¹⁴⁰ Furthermore, individual crystals also may spiral on themselves.¹⁴¹ While the three-dimensional arrangement of crystals associated with enamel rods is reasonably well understood, the same cannot be said for crystals in interrod enamel. Indeed, interrod enamel has been the subject of relatively few studies. Other more classical aspects of the rod and interrod enamel structure as they relate to rod striations, rod sheaths, Hunter-Schreger bands, striae of Retzius, perikymata, and enamel tufts, spindles, and lamellae are covered in two recent reviews.^{1,135}

The unit cell of enamel crystals is generally defined as that of hydroxyapatite [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$], but it is well known that these crystals can undergo substitution with ions such as magnesium, fluoride, and carbonate.^{1,142} Young enamel is relatively rich in carbonate, which can replace either the phosphate or hydroxyl group at the surface or inside the crystals.^{25,142-145} As the crystals age, the CO_3/Ca ratio decreases and there is a change in the sites of substitution within the crystal lattice.^{25,143-145} These changes most probably reflect the evolving microenvironment in which enamel develops, and may be related to possible phases of calcium phosphate occurring during apatite formation.^{25,144,146-151} Carbonate makes enamel more soluble in acid, and its presence has been implicated in the central dissolution of crystals seen in carious lesions.^{1,152} This concept of central dissolution, although widely accepted, has also been interpreted to reflect physical properties related to the shape of the crystals rather than to their composition.^{136,141}

While the basic c-axis shape and trajectory of enamel crystals is widely accepted, a controversy exists concerning the true a- to b-axis shape of older crystals. To date, the most widely held view is that individual enamel crystals begin as elongated thin plates that become hexagonal in cross-sectional outline

as the crystals mature.^{1,133,153-157} It has been proposed that the hexagonal outline observed in thin sections of enamel by transmission electron microscopy actually may represent two-dimensional projections of obliquely cut, three-dimensional, rhombohedral crystal segments.¹⁴¹ This interpretation, based on the fundamental principle that transmission electron microscope images represent shadows cast on the viewing screen or recording medium, predicts that crystals having a hexagonal shape should project as octagonal profiles when sectioned obliquely.¹⁴¹ Octagonal crystal profiles are almost never seen in routine thin sections of enamel, despite the fact that the extremely long, tortuous trajectory of enamel crystals would favor them being cut obliquely very often.¹⁴¹ Recently it has been argued that segments of hexagonal-shaped crystals would in many cases project as hexagons and occasionally as octagons.¹⁵⁶ It has been proposed that these latter profiles are rarely observed in sections because "low-contrast edges" make their eight corners difficult to visualize.¹⁵⁶ There is also evidence that as much as 2.2° of variation can exist between the c-axis orientation of the unit cell and the long axis of the crystals themselves.¹⁵⁸ This would imply that a lattice image characteristic of hexagonal symmetry¹⁵⁹ could in fact be imaged onto a profile projected from a crystal not in perfect cross section. Ultimately, it should be noted that as enamel crystals mature they expand substantially and press against neighboring crystals.^{139,160} Since they must conform to adjacent crystals within the available volume, their outline will become less regular over time.^{160,161}

In most mineralized tissues, an organic component of the matrix is believed to act as a template for orienting crystal growth and is intimately associated with the mineral phase.¹³³ Which EPs serve this purpose is unclear at present, but morphological studies of young enamel crystals, following demineralization, reveal a residual proteinaceous "ghost" which has the same shape and orientation, and virtually occupies the same space as the crystal itself.^{162,163} While the coexistence of protein and mineral in crystals with only a few lattices in thickness is consistent with the notion that some EPs reside at the surface of crystals,^{35,133,163,164} it has been argued that the "elastic band" profiles obtained following demineralization of older crystals are too small and indeed appear to be located within the crystal itself, a situation which is questionable from the crystallographic point of view.¹⁶⁵

The issues of how enamel crystals are seeded and grow to their unusually large dimensions are largely unanswerable at present, in part because of incomplete information concerning the nature and exact chemical composition of all matrix proteins being secreted by ameloblasts, and in part because of lack of knowledge about the true functional meaning of the rapid modulation cycles that ameloblasts display during the maturation stage of amelogenesis. In regard to crystal seeding, the debate has revolved around two diametrically opposite views, that is, whether ameloblasts themselves secrete the "nucleating factors" needed to induce development of crystallites locally within the rod and interrod regions of enamel (active seeding), or whether it occurs as

an extension in the growth of crystallites seeded originally within dentin which mineralizes immediately before the initial layer of enamel is produced (passive seeding).^{129,133,134,146,166,167} When first reported, the enamelin were of great interest because these acidic EPs appeared to contain the accessory chemical groups, such as phosphate and sugar, considered necessary to induce mineral deposition.⁵¹ This issue is presently much less clear, however, since there has been no universal consensus as to the exact biochemical properties of proteins defined as "enamelin",⁶³⁻⁶⁶ and proteins within the hydrophobic amelogenin group have since been reported to contain phosphate and sugar groups, to possess hydrophilic regions in addition to long hydrophobic regions within their primary structure, and to possess calcium ion-binding and hydroxyapatite-binding properties.^{34,35,129} In addition to nucleating crystals, part of the seeding process presumably also involves appropriate three-dimensional spacing of nucleation sites to take into account the future growth in thickness and width the crystals will undergo during the maturation stage of amelogenesis.

Once enamel crystals are seeded, they are then believed to increase in size by two tightly controlled processes. The first process involves growth in the length of the crystallites. This presumably happens continuously throughout the secretory stage, as ameloblasts secrete large amounts of new EPs and build up the enamel layer by appositional growth. Since a distinct unmineralized preenamel layer is not observed between the ameloblast and the extracellular matrix in well-preserved specimens,^{168,169} the plasma membrane of ameloblasts must be pressed directly against the rod and interrod growth sites where the crystals are elongating.¹⁴⁴ The three-dimensional trajectories of the growing ends of enamel crystals, and ultimately regions of rod and interrod enamel, are likely therefore, greatly influenced by the chemical nature of proteins embedded within the plasma membranes of ameloblasts or deposited near the surface of the membranes at these sites.^{129,133} The second process occurs later in time and involves expansion in the thickness and width of the enamel crystals.^{133,153-155} The central dispute here is whether this growth occurs slowly and progressively over time (linear growth),^{44,153-155} or if there is an interval during the maturation stage of amelogenesis when the rate of mineral accretion at the surface of the crystal accelerates rapidly to completion (exponential growth).⁵⁰ There is fairly wide agreement that enamel crystals can grow in thickness and width only if other components present in the enamel layer are removed to provide the free space, or volume, into which the crystals can expand.^{50,170} Available evidence indicates that the disposable components of enamel are the amelogenins and water.^{35,50} It is debated, however, whether growth in thickness and width of the crystals directly stimulates breakdown of the amelogenins, or if the amelogenins must first be degraded by proteinases in order to allow growth in thickness and width of the crystals.³⁵ Irrespective of which event happens first, there seems to be good agreement that the mineral binding properties of the EPs and their water

solubility likely exert considerable local control over the rate of crystal growth in thickness and width, until these proteins begin to disappear.^{35,129,170} Ultimately, it must be remembered that the formation of crystals of hydroxyapatite with unusually large dimensions is a process that takes many weeks to complete,⁵⁰ as opposed to only hours, as seen in those hard tissues using a mineralization front to produce small plate-like crystals of hydroxyapatite in a collagenous matrix.¹³³ It is now well established that ameloblasts rhythmically alter their morphology at the surface of enamel over the same period of time that the enamel crystals expand in thickness and width.^{37,38} Such cyclic modulations represent a biological process unlike anything seen in any other of the hard tissues. This phenomenon presumably, therefore, provides the unique mechanism which allows additional mineral to be deposited continuously over the long periods of time at the surface of maturing enamel crystals.

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