Scanning Microscopy

Volume 7 | Number 1 Article 18

12-17-1992

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Scanning Microscopy, Vol. 7, No. 1, 1993 (Pages 165-175) 0891-7035/93$5.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

HIGH-RESOLUTION SCANNING ELECTRON MICROSCOPY OF RAT INCISOR AMELOBLASTS

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(Received for publication November 4, 1992, and in revised form December 17, 1992)

Abstract

The internal three-dimensional organization of secretory and maturation stage ameloblasts was examined using field emission scanning electron microscopy. Particular attention was given to the structure of the Golgi apparatus, the distribution of smooth membrane tubulo-vesicular elements and their relationship with endosomal/lysosomal components. Rat incisors were fixed by intracardiac perfusion with glutaraldehyde, decalcified and divided into segments. The tissues were cryoprotected with glycerol and freeze-fractured. They were then macerated in osmium, and after conductive staining with osmium/tannic acid, the samples were critical-point dried and sputtered with gold. High-resolution scanning electron microscopy showed that ameloblasts contain a well-developed Golgi apparatus, even in the maturation stage where cells are generally believed not to be actively involved in protein secretion. Smooth tubulo-vesicular structures formed a complex network which extended throughout the cell. In secretory stage ameloblasts, this network reached into Tomes' process and consisted of small and large tubules, and distended portions. The smaller tubules radiated from a central core of organelles towards the plasma membrane. Numerous lysosomal/endosomal elements were observed in the Golgi region, and in some cases smooth tubular portions were seen at the surface of multivesicular bodies. These data show that high-resolution scanning electron microscopy can be applied to correlate three-dimensional structural detail with the secretory and resorptive functions of ameloblasts.

Key Words: High-resolution, field emission, scanning electron microscopy, ameloblasts, Golgi apparatus, tubulo-vesicular elements, endosomal/lysosomal system.

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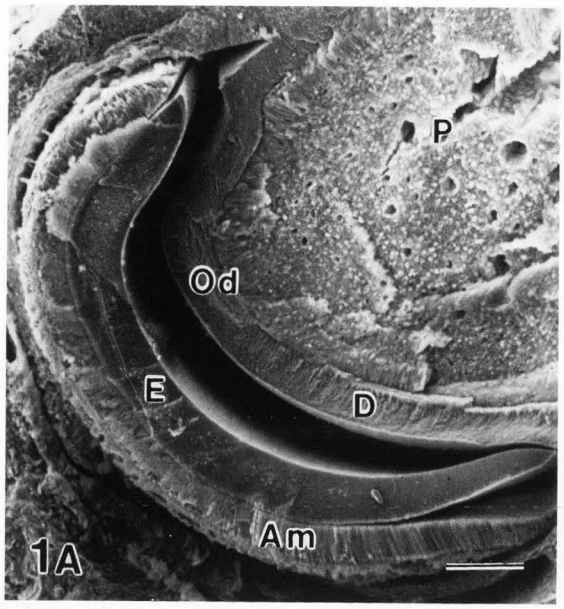
Introduction

During enamel formation, ameloblasts produce an extracellular matrix which initially may play a role in seeding, and subsequently in regulating the formation of extremely long and intricately organized apatite crystals. This organic matrix is then degraded and almost totally removed in order to allow final growth of the crystals. The interplay between these formative and degradative events ultimately leads to the creation of the hardest tissue of the body (reviewed in Nanci and Smith, 1992).

The morphology of ameloblasts undergoes important changes throughout their life cycle, reflecting specific functions associated with each stage of amelogenesis. There have been several studies detailing the ultrastructure of ameloblasts, however, few of them have dealt with the three-dimensional internal organization of these cells. Indeed, despite recent developments in the application of scanning electron microscopy to visualize cell structure (reviewed in Tanaka, 1989), there has been, to our knowledge, only one systematic high-resolution scanning electron microscope (SEM) study of the organization of organelles in ameloblasts from different stages (Ohmi, 1987), and two reports on the three-dimensional morphology of the distal portion of maturation stage ameloblasts (Kishino *et al.*, 1989, 1991). We have taken advantage of the high-resolution capability of the field emission SEM to reexamine the spatial organization of organelles in secretory and maturation stage ameloblasts in light of recent findings on the formation and degradation of enamel proteins (reviewed in Nanci and Smith, 1992), and on the nature of the endosomal compartment (Hopkins *et al.*, 1990). The continuously erupting rat incisor was used as a model since all the stages of amelogenesis are found in a single tooth (reviewed in Leblond and Warshawsky, 1979).

Materials and Methods

Male Wistar rats (Charles River, St-Constant, QC) weighing approximately 100 g were anesthetized with chloral hydrate (0.4 mg/g body weight) and perfused through the left ventricle into the aorta with lactated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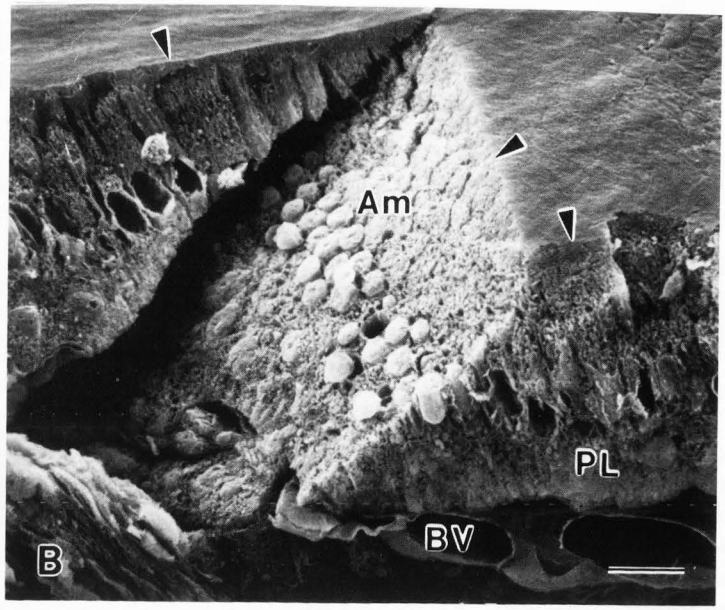
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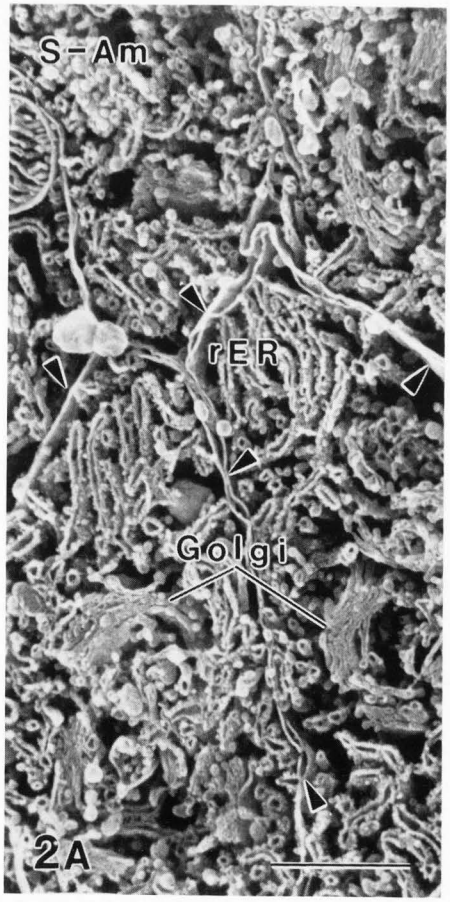
S-Am
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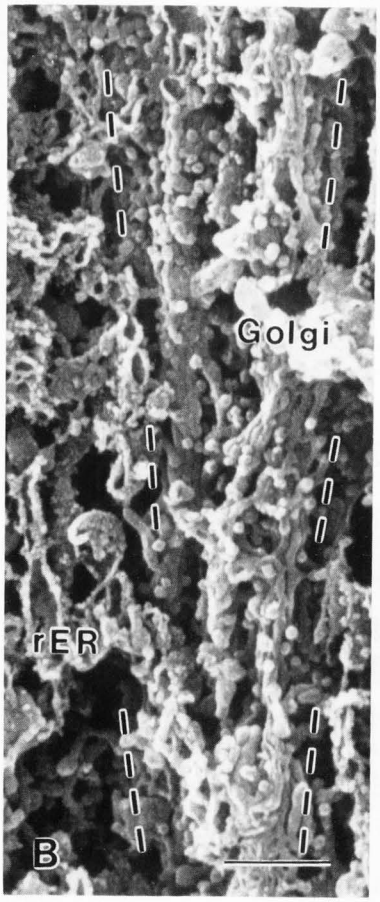
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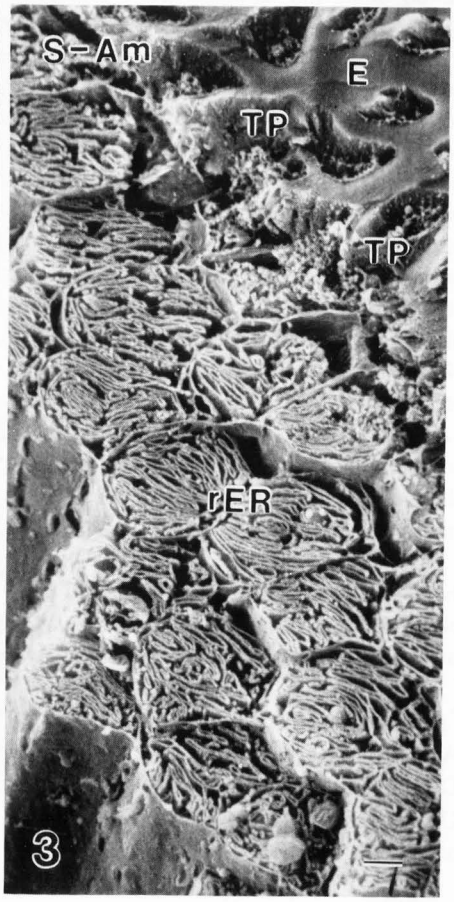
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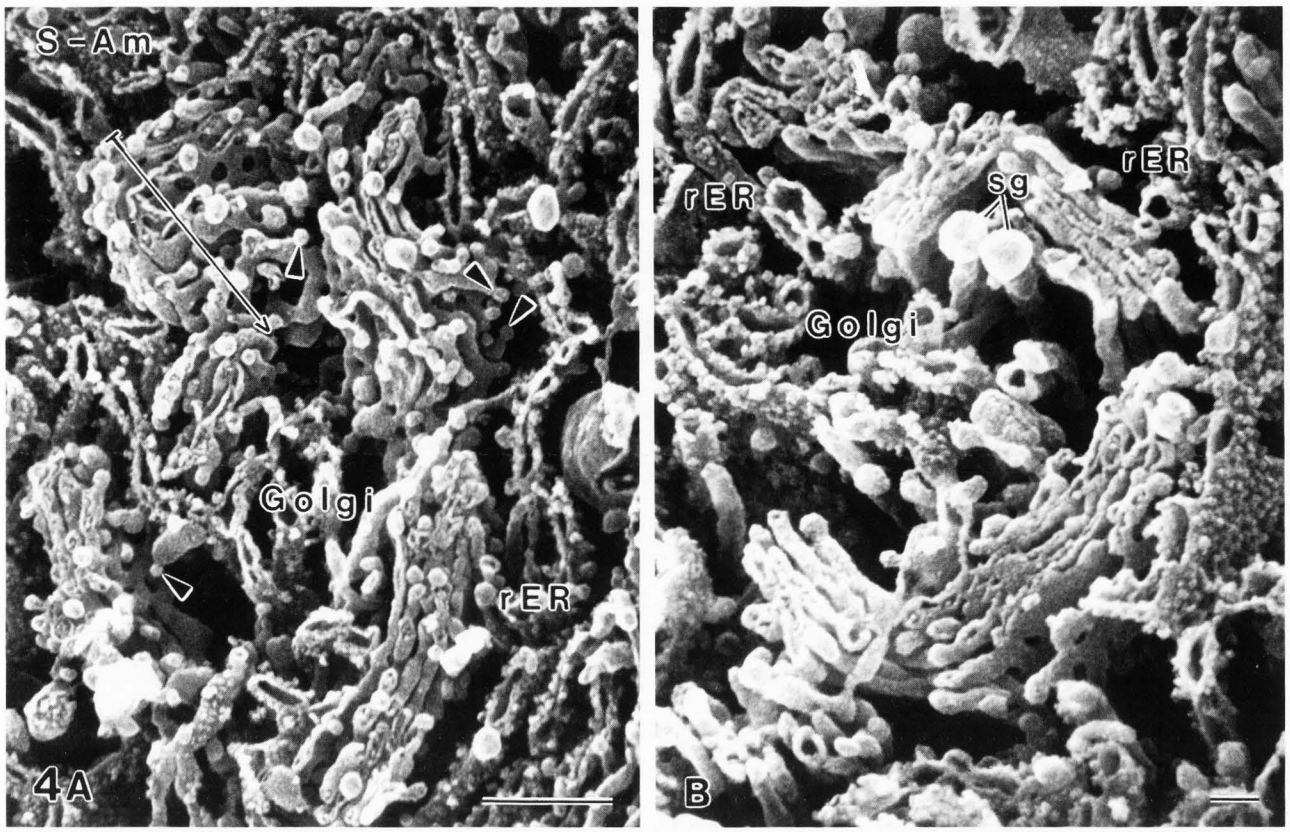


Figures 4A, 4B (above): The Golgi apparatus (Golgi) of secretory stage ameloblasts (S-Am) is surrounded by cisternae of rough endoplasmic reticulum (rER) which sometimes reach deep among the stacks. The saccules from the *cis* to the *trans*-face (long arrow) show fenestrations of different size. The *trans*-face is characterized by the presence of a widely fenestrated saccule or tubular reticulum. Some of the saccules also show small vesicular blebs (arrowheads). sg: secretory-like granule. Bars = A: 0.5 μm; B: 100 nm.

Figure 2 (on the facing page): (**A**) Cross-sectional and (**B**) longitudinal views of the Golgi region of secretory stage ameloblasts (S-Am). The Golgi apparatus (Golgi) forms a compact cylindrical structure along the length of the cell and is surrounded by rough endoplasmic reticulum (rER). Note the polyhedral outline of ameloblasts in cross-section (arrowheads). Bars = A: 1 μm; B: 0.5 μm.

Figure 3 (on the facing page): The portion of the supranuclear compartment of secretory stage ameloblasts (S-Am) distal to the Golgi apparatus is occupied mostly by longitudinally-oriented cisternae of rough endoplasmic reticulum (rER). TP: Tomes' process; E: enamel. Bar = 1 μm.

Ringer's solution (Abbott Laboratories, Montréal, QC) followed by 0.5% glutaraldehyde in 0.15 M phosphate buffer (PB; pH 7.4) for 10 minutes. The hemimandibles were further fixed by immersion in the same fixative for an additional hour and then decalcified in 4.13% EDTA for 14 days (Warshawsky and Moore, 1967). Tissue segments from the secretory and maturation (first 4-6 mm) stages were prepared using a molar reference line (Smith and Nanci, 1989a). The samples were cryoprotected with 30% glycerol in PB, frozen in partially solidified Freon 22 cooled by liquid nitrogen and fractured under liquid nitrogen using a precooled razor blade (Kan and Nanci, 1988). The fractured tissues were deglycerinated in PB, postfixed in 1% osmium tetroxide in PB, macerated for 96 hours in 0.1% osmium in PB and processed for conductive staining using osmium/tannic acid (Inoué, 1986; Tanaka, 1989). They were then dehydrated in acetone, critical point dried using CO_2, and sputtered with a thin coat of gold. The specimens were examined in a JEOL JSM-6300F field emission SEM operated at an accelerating voltage of 15 kV.

For immunocytochemistry, rat incisor enamel organs were fixed with 1% glutaraldehyde and conventionally processed for Epon embedding. Thin sections were

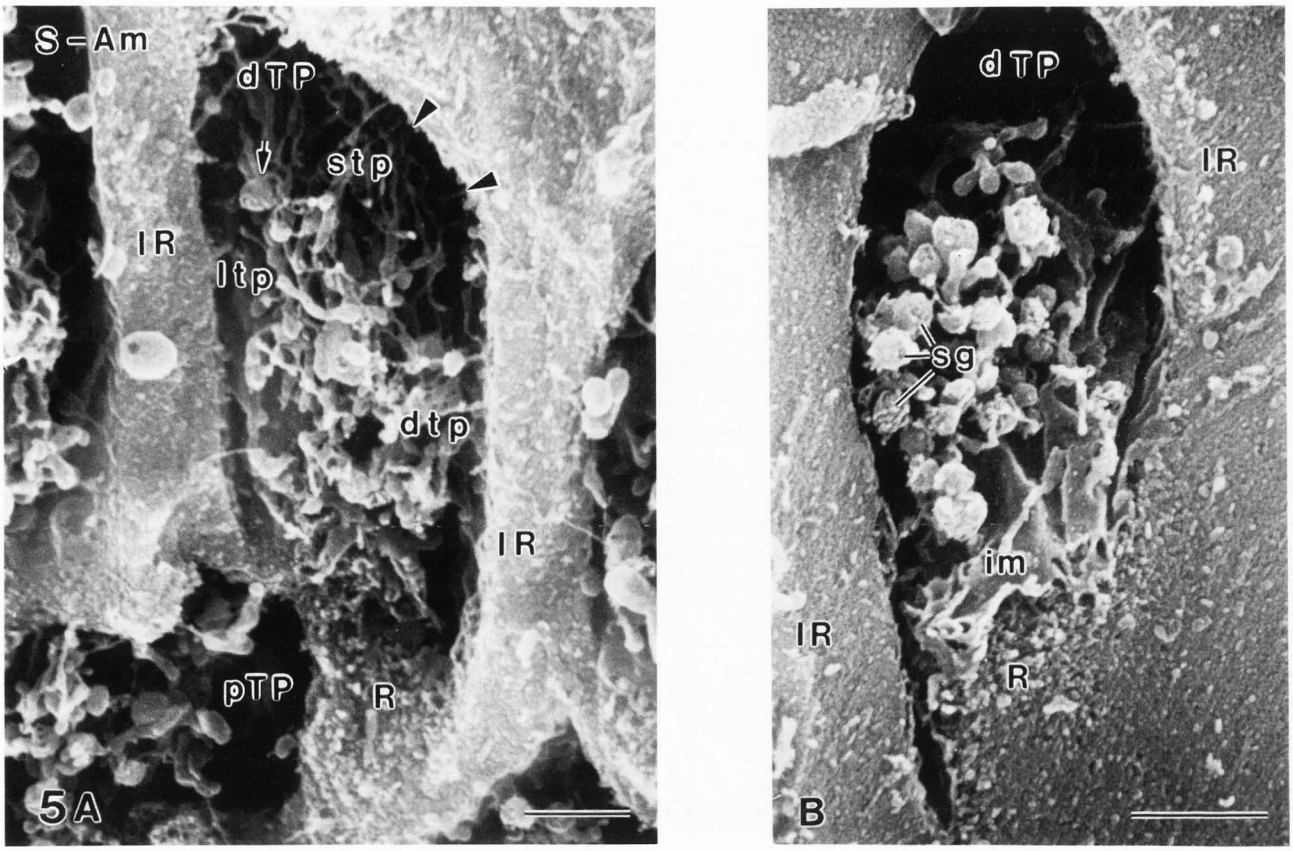


Figure 5: (**A**) Smooth tubular elements extend into both the proximal (pTP) and distal (dTP) portions of Tomes' process. In the distal Tomes' process, this tubular network is clearly seen to be composed of small (stp) and large (ltp) tubular, and distended (dtp) portions. The smaller tubules seem to radiate towards the cell surface (arrowheads), and occasionally, vesicular structures / secretory-like granules appear to be attached to some of them (small arrow). (**B**) The numerous secretory granules (sg) present in Tomes' process are intimately associated with the smooth tubular network. im: infolded membrane at rod secretion site; IR: interrod enamel; R: rod enamel; S-Am: secretory stage ameloblasts. Bars = 1 μm.

incubated with an antibody to mouse amelogenins followed by the protein A-gold complex, as previously described (Nanci *et al.*, 1987).

Results

Secretory stage ameloblasts

Although fracturing of tissues was performed along well-defined axes of the incisor (Fig. 1A), the plane of fracture was not always predictable. Cross, tangential and longitudinal views of ameloblasts were obtained (Fig. 1B). In secretory stage ameloblasts (Figs. 2-5), the Golgi apparatus was well-developed and, judging from a composite image derived from all three planes of fracture, it was organized into a compact cylindrical structure occupying much of the supranuclear compartment (Fig. 2B). The closely-apposed Golgi saccules displayed a progressive increase in the size of the fenestrations from the *cis* to the *trans* face and some saccules also showed small vesicular blebs (Fig. 4). Certain planes of view revealed the presence of a widely fenestrated saccule/tubular network on the *trans*-most aspect of the Golgi stacks (Fig. 4A). The Golgi compartment was surrounded by rough endoplasmic reticulum (Figs. 2A, 4) and occasionally cisternae penetrated deep into the central core of this organelle (Fig. 4B). Small, secretory-like granules were present on the *trans* face of the Golgi stacks (Fig. 4B). The remaining portion of the supranuclear compartment, distal to the Golgi apparatus, was mostly occupied by rough endoplasmic reticulum organized as longitudinally-oriented sheaths (Fig. 3). The ameloblasts contained an extensive network of smooth tubulo-vesicular elements which surrounded the nucleus and extended across the supranuclear compartment into Tomes' process (Fig. 5A). In the distal portion of Tomes'process, various components

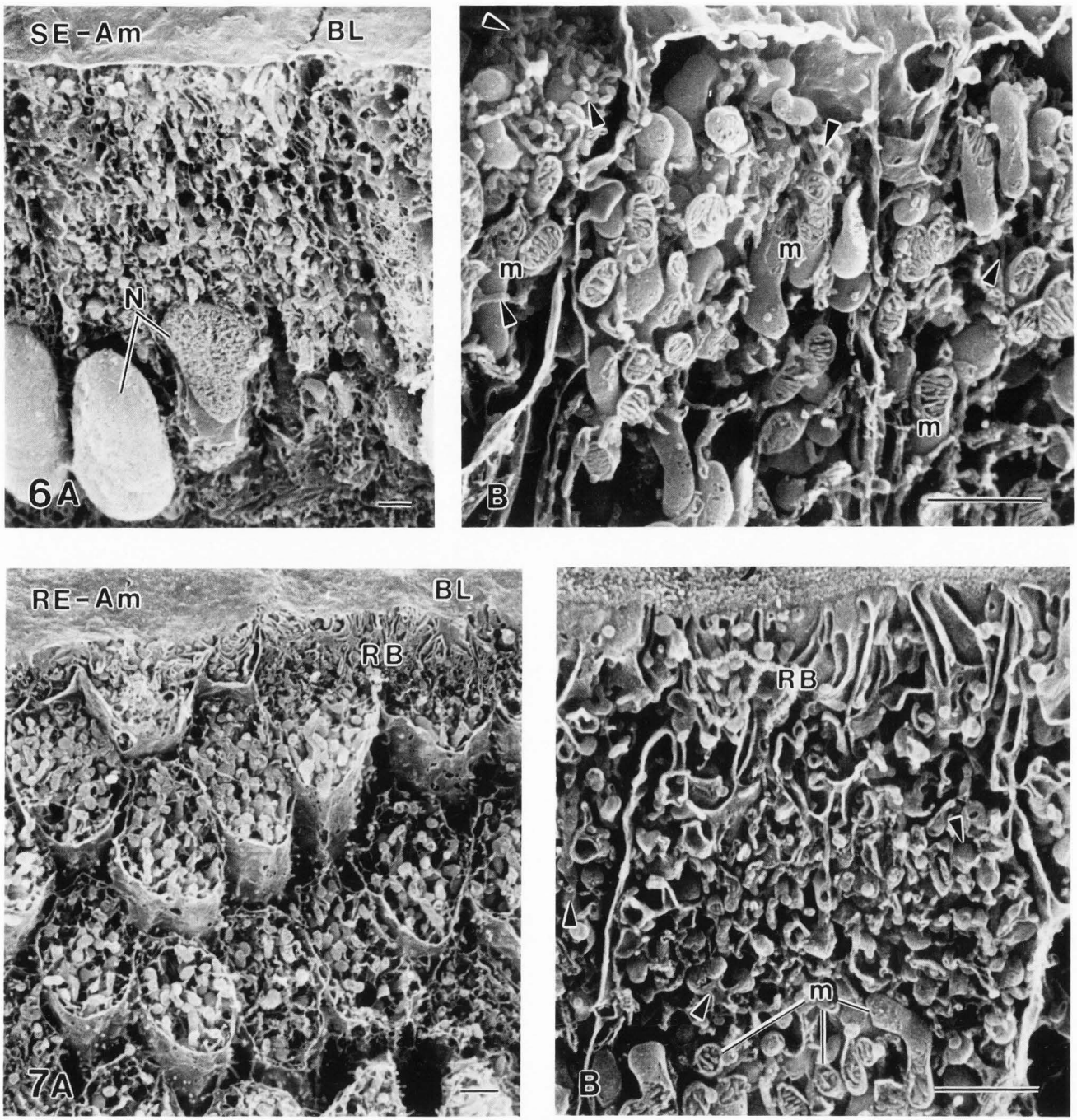
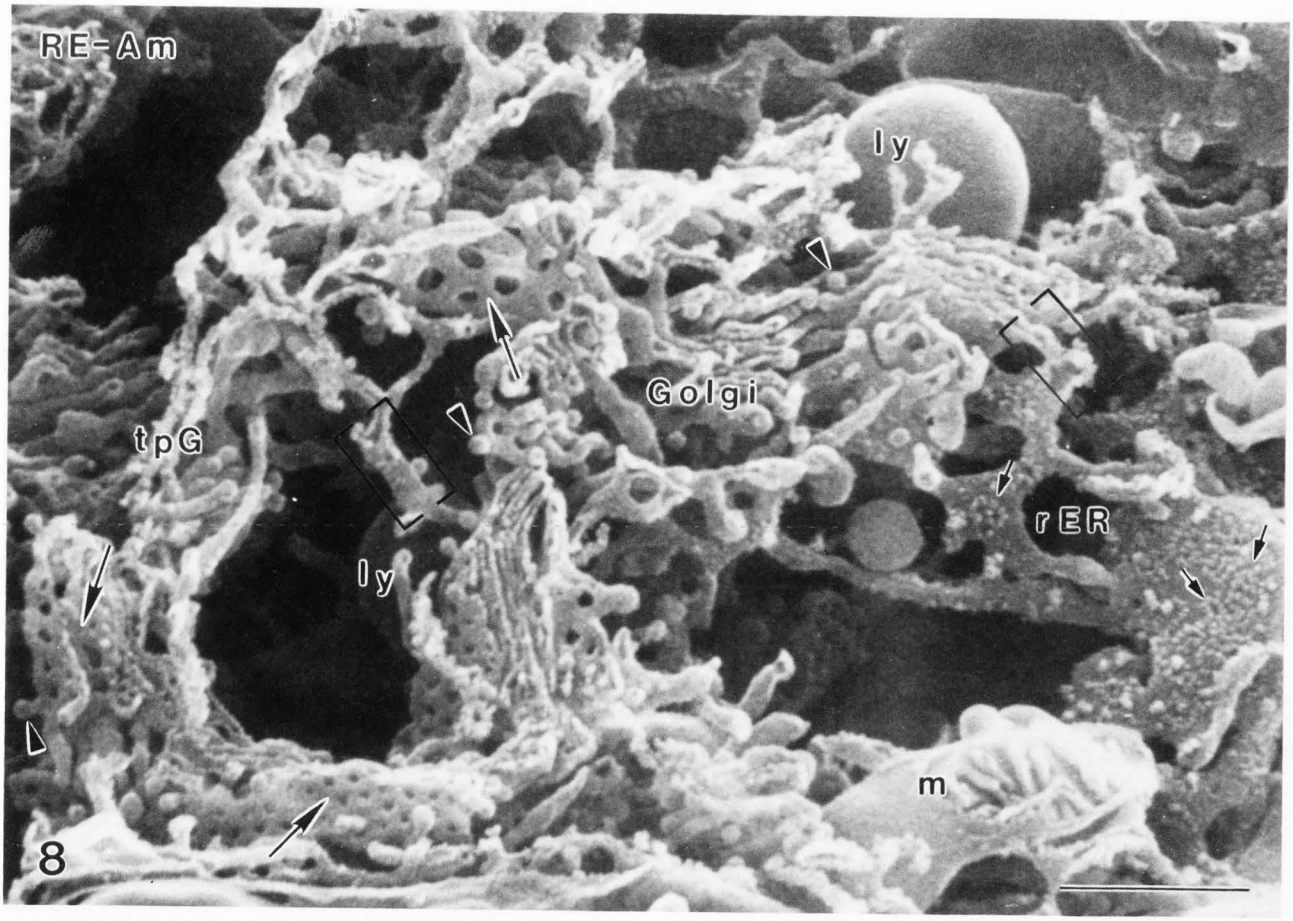


Figure 6: (**A**) Low-magnification view of longitudinally-fractured smooth-ended maturation stage ameloblasts (SE-Am). A basal lamina-like structure (BL) covers the apical surface of cells in this stage. Some nuclei (N) are intact and nuclear pores can be seen on them while others are fractured open, thus exposing the chromatin. (**B**) At higher magnification, smooth tubules (arrowheads) are seen to extend up to the apical surface of the ameloblasts and to form an extensive network intermingling with numerous mitochondria (m). Bars = 1 μm.

Figure 7: (**A**) Low-magnification micrograph of tangentially-fractured ruffle-ended maturation stage ameloblasts (RE-Am) showing the organization of organelles at different levels of the supranuclear compartment. (**B**) A higher magnification of the ruffled border (RB) of these cells. The membrane infoldings of the ruffled border are associated with smooth-membrane polymorphic structures (arrowheads) at the base of which mitochondria (m) accumulate. BL: basal lamina. Bars = 1 μm.


RE-Am
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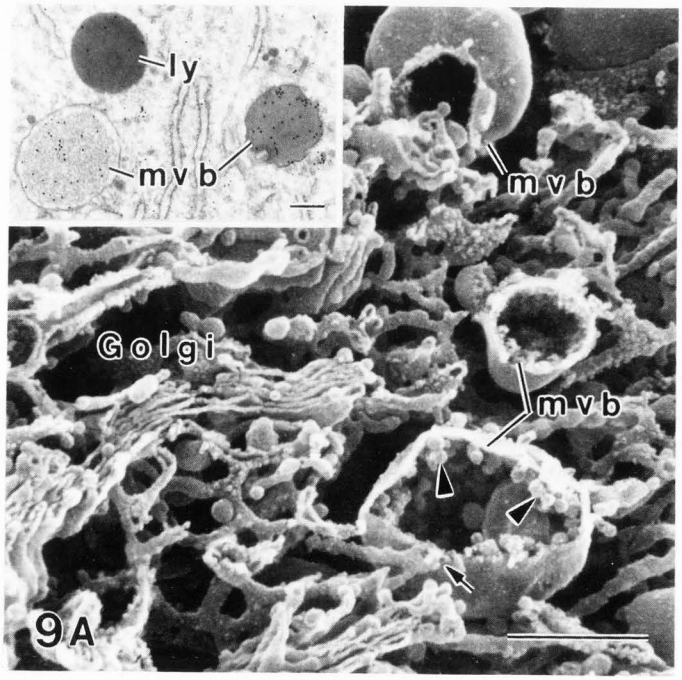
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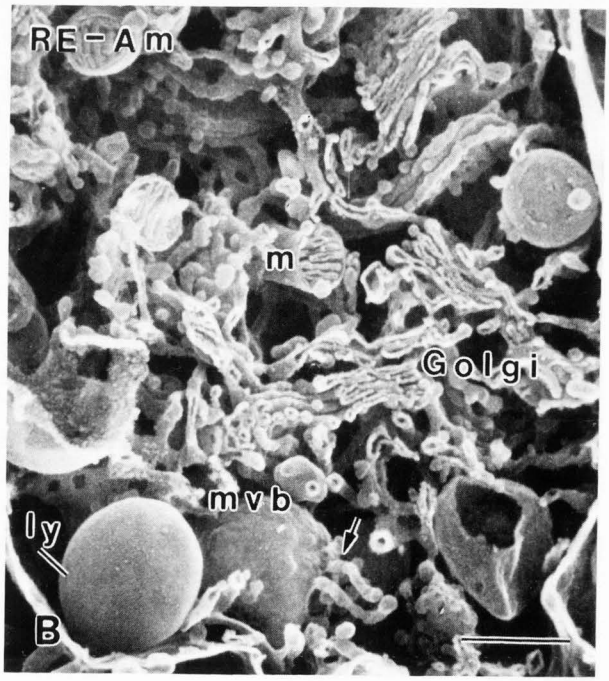
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Figure 8 (on the facing page): The Golgi apparatus (Golgi) in maturation stage ameloblasts forms a spheroidal mass situated just above the nucleus. The various stacks are interconnected by tubular portions (tpG). The various saccules (large arrows) show fenestrations which vary in size from the *cis* to the *trans* face of the Golgi apparatus. Vesicular blebs (arrowheads) are also seen on some of the saccules. Portions of rough endoplasmic reticulum (rER), with few or no ribosomes (small arrows), are intimately associated with the Golgi stacks, in some cases giving the impression of a continuity between rER and Golgi elements (brackets). ly: lysosome; m: mitochondria; RE-Am: ruffle-ended ameloblast. Bar = 0.5 μm.

Figures 9A, B (on the facing page): Golgi region (Golgi) in ruffle-ended maturation stage ameloblasts (RE-Am). Numerous lysosomal/endosomal elements are present in this region. Lysosomes (ly) seem to have a smoother surface while multivesicular bodies (mvb), recognizable by the presence of typical small vesicles in them (arrowheads), have a more rugged surface. Note the presence of smooth tubular portions (small arrows) at the surface of some multivesicular bodies. Inset: transmission electron microscope immunocytochemical preparation showing the presence of enamel proteins in mvbs, and a difference in surface contour between a dark lysosome and multivesicular bodies. m: mitochondria. Bars = A, B: 0.5 μm; inset: 0.25 μm.

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of this network were readily distinguished and these consisted of small and large tubules, and distended portions (Fig. 5A). The smaller tubules radiated towards the plasma membrane opposite the enamel rod growth site (Fig. 5A). Although, smooth tubular elements were closely associated with secretory-like granules (Fig. 5B), relatively few direct connections with vesicles or granules were observed.

Maturation stage ameloblasts

In the maturation stage, both smooth- and ruffle-ended ameloblasts were examined (Figures 6-9). The smooth-ended cells were characterized principally by a smooth apical surface and mitochondria dispersed throughout the cytoplasm (Fig. 6). The mitochondria often reached the cell surface and intermingled with an extensive smooth tubulo-vesicular network present in the apical portion of the ameloblasts (Fig. 6B). Deep membrane infoldings were found at the apical surface of ruffle-ended ameloblasts (Fig. 7), and these infoldings seemed to form or intermix with an extensive network of polymorphic structures (Fig. 7B). Numerous mitochondria accumulated at the base of the ruffle border (Fig. 7B). In both smooth- and ruffle-ended ameloblasts, particularly in the early part of the maturation stage, the Golgi apparatus was still relatively extensive and formed a spheroidal mass situated just above (distal to) the nucleus (Fig. 8). The various Golgi stacks composing this mass were interconnected by tubular portions (Fig. 8). The saccules, from the *cis* to the *trans* face, showed fenestrations of variable size (Fig. 8). Cisternae of rough endoplasmic reticulum were found in close proximity to the Golgi apparatus and some of them, showing few or no ribosomes, intermingled with the saccules (Fig. 8). Large, lysosomal-like elements, with either a smooth or rugged surface, were also observed in the Golgi region (Fig. 9). Some of the elements with a rugged surface were identified as multivesicular bodies by the presence of "typical" small vesicles within them, and occasionally, smooth tubular portions were also seen at their surface (Fig. 9B).

Discussion

The present study indicates that high-resolution scanning electron microscopy is advantageous for examining and correlating three-dimensional structure with the function of ameloblasts at the different stages of amelogenesis. The information obtained with this approach is normally otherwise obtained from more laborious serial sectioning and three-dimensional reconstruction. High-resolution scanning electron microscopy also generally provides more spatial information than replica methods (Haggis, 1992) or high-voltage transmission electron microscope examination of thick sections. Although visualization of organelles in the SEM relies largely on the extraction of protein by maceration procedures (reviewed in Lea *et al.*, 1992), it is hoped that future developments will allow integration of intracellular three-dimensional structure with cytochemical and immunocytochemical labeling.

Maturation stage ameloblasts have for long been perceived as post-secretory cells, however, recent biochemical, immunocytochemical and radioautographic data have clearly shown that these cells are still capable of actively synthesizing and secreting extracellular matrix proteins (Nanci *et al.*, 1987; Inage *et al.*, 1989; Nanci *et al.*, 1989; Smith and Nanci, 1989b). In the early part of the maturation stage, these cells show immunoreactivity for enamel proteins in secretory granules and in the Golgi apparatus (Nanci *et al.*, 1987; Inage *et al.*, 1989; Nanci *et al.*, 1992), and have been found by quantitative radioautography to secrete almost as much protein as ameloblasts which form the initial layer of enamel (Smith and Nanci, 1989b). Indeed, the present study shows that maturation stage ameloblasts possess a well developed Golgi apparatus, adding morphological

support to these findings. In ameloblasts from both the secretory and maturation stages, and as is generally the case for most cells, the saccules forming the stacks show a progressive increase in the size of fenestrations from the *cis* to the *trans* face of the Golgi apparatus (reviewed in Rambourg and Clermont, 1990). The *trans* face is characterized by the presence of a tubular reticulum which may correspond to part of the *trans*-Golgi network (Griffiths *et al.*, 1989; Geuze and Morré, 1991). A Golgi apparatus-endoplasmic reticulum-lysosomes system (GERL; Novikoff, 1976), which may have some relationship to the *trans*-Golgi reticulum (Geuze and Morré, 1991), was demonstrated in ameloblasts at various stages of amelogenesis using acid phosphatase cytochemistry (reviewed in Ozawa *et al.*, 1983). Previous high-resolution SEM studies have suggested the existence of interconnections between the rough endoplasmic reticulum and Golgi elements in nerve cells (Tanaka *et al.*, 1986; Tanaka and Fukudome, 1991). In the present study, cisternae of rough and/or transitional endoplasmic reticulum were observed to penetrate deeply among Golgi stacks and approximate some of the saccules. Although some of the SEM images give the impression of continuity between rough or transitional endoplasmic reticulum and Golgi elements, interconnections between these organelles are not readily apparent. Since it is widely believed that transport from the rough endoplasmic reticulum to the Golgi apparatus occurs via vesicles (reviewed in Pryer *et al.*, 1992), elucidation of whether interconnections between these two organelles are a general occurrence, and whether they represent a major route for protein sorting, will require further studies. More specifically, with respect to high-resolution scanning electron microscopy, systematic analysis of multiple fields and fracture planes using stereo-microscopy in various cell types will be required. The possibility that small transfer vesicles are lost during maceration and that interconnections are formed artefactually during tissue processing (including confluence of the metallic coating on adjacent structures) must be taken into consideration when interpreting the SEM images (discussed in Tanaka and Fukudome, 1991).

It is now well documented that ameloblasts possess an extensive smooth membrane tubulo-vesicular network which extends throughout the cell (Ozawa *et al.*, 1983; Ohmi, 1987). This network is particularly evident at the apical portion of smooth-ended maturation stage ameloblasts and in Tomes' process of secretory stage ameloblasts where it is intimately associated with secretory-like granules. Although images were observed which suggest that occasional granules may be attached to smooth tubular elements in secretory stage ameloblasts, perhaps corresponding to the tail-like extensions observed on some dark-staining granules in Tomes' process (Nanci and Warshawsky, 1984), there is, at present, no definitive evidence that secretory granules originate from, or release their content via this network. Release of enamel proteins may instead be associated with channels formed by membrane infoldings present at secretion sites (Simmelink, 1982; Nanci and Warshawsky, 1984). Indeed, transmission electron microscope studies following zinc iodide-osmium tetroxide (ZIO) impregnation also suggested that some granules seem to be connected to the tubulo-vesicular system, but tilting of the sections revealed that this apparent continuity was caused by overlapping of superimposed structures (Uchida and Warshawsky, 1992).

Portions of the smooth tubular network radiate towards the cell membrane of Tomes' process opposite the secretory sites. These portions generally appear thinner and may correspond to the tubular profiles previously described (Nanci and Warshawsky, 1984). Although a previous SEM study reported that smooth endoplasmic reticulum is connected to the rough endoplasmic reticulum and actually "stretches from the rough endoplasmic reticulum" to form a network into Tomes' process (Ohmi, 1987), we have not encountered such connections. Indeed, cytochemical studies with ZIO show that reactive smooth membrane tubules extend from the Golgi apparatus, across the rough endoplasmic reticulum, and into Tomes' process (Ozawa *et al.*, 1983).

The identity and precise role of tubulo-vesicular elements in ameloblasts remain to be clarified. Based essentially on the continuity between Golgi-GERL *(trans*-Golgi network ?)-ZIO-reactive smooth tubular elements, and the distribution of acid hydrolases in these components, it has been proposed that smooth tubular elements may play an important role in endocytosis (Ozawa *et al.*, 1983). The association of such elements with the apical plasma membrane and with multivesicular bodies of ameloblasts is consistent with this notion. Likewise, it has been shown that ruffle-ended ameloblasts have a network of tubular lysosomes, extending throughout the cell, which are most abundant at a time when there is active protein removal from the enamel layer (Salama *et al.*, 1989; 1990). Recent studies have suggested that the endosomal system consists of a continuous tubular reticulum leading into multivesicular bodies (Hopkins *et al.*, 1990), a concept supported by the presence of smooth tubular portions at the surface of some multivesicular bodies. Furthermore, the present study may offer a possible morphological distinction between lysosomes (dark type) and multivesicular bodies. Lysosomes generally appear to have a smoother surface whereas the surface of multivesicular bodies seems to be more rugged. Alternatively, it has been proposed that some smooth tubular elements may play a role in calcium handling (Nanci and Warshawsky, 1984; Takano *et al.*, 1989; 1990). In-

deed, it has been found that secretory stage ameloblasts contain a tubulo-vesicular network which stains with glyoxal bis (2-hydroxyanil), a calcium stain, and in which can also be found calcium deposits (Takano *et al.*, 1989; 1990).

Acknowledgements

The authors wish to thank G. Lambert for photographic reproductions and Dr. T. Inoué (Tottori University, Yonago, Japan) for advice concerning high-resolution SEM. The antibody to mouse amelogenins was kindly provided by Prof. H.C. Slavkin, Univ. Southern California, Los Angeles. This study was supported by the Medical Research Council of Canada.

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Discussion with Reviewers

D.R. Eisenmann: In the discussion, reference is made to previously reported channels formed by membrane infoldings at secretion sites in secretory ameloblasts. Were these structures observed in the present SEM study? Can they be distinguished from the smooth tubular network?

Authors: Membrane infoldings associated with secretory sites were indeed observed in the present study (see figure 5B). When seen in three dimensions, these infoldings are veil-like in appearance, and although some of their deeper portions may form more discrete channels, these are easily distinguished from the distinctly tubular smooth membrane profiles.

M. Goldberg: It is well known that during conventional (aldehyde) fixation (Plattner, Cell Biol. Int. Rep. **5**:435-459, 1981) exocytosis occurs. As the tissue was fixed before freezing and therefore lost part of the secretory vesicles, how confident can the authors be on the reliability of the appearance of the content of Tomes' process?

Authors: It is well known that chemical fixation may introduce changes in the composition and/or structure of tissues. However, in many cases these changes occur at the molecular level and do not necessarily affect the overall structural appearance of organelles. For instance, in cryofixed tissues, extracellular matrices or antigenicity of molecules may differ, but the cellular architecture is not necessarily dramatically different. Indeed, the internal and external architecture of cryoprocessed ameloblasts in transmission electron microscope preparations does not differ significantly from that of the conventionally prepared tissues (Nanci, Kogaya and Kawaguchi, manuscript in preparation). As regards the article by Plattner that you reference, again the most dramatic changes induced by chemical fixation and cryoprotecting agents, which are discussed in this review, occur at the molecular level (e.g., membrane protein). It will nonetheless be of interest to compare the present results with that of cryoprocessed tissues observed in the SEM. In this regard, recent developments in low-temperature field emission scanning electron microscopy may prove advantageous.

M. Goldberg: Interrod enamel appears in Figure 5A as a very compact and homogeneous structure. In Figure 5B fiber-like structures are seen, as is the case in thin sections and in most other reports including investigations using the SEM and osmium maceration (Salomon *et al.*, Scanning Microsc **5**: 509-517, 1991). Can the authors comment on the appearance of the forming interrod enamel of their Figure 5A: swelling, influence of glycerol or any other interaction. Does this cast doubt on the actual preservation of intracellular organelles?

Authors: Since the original treatment of both tissue samples was the same and as discussed by Salomon *et al.* (reference you cite), the difference in appearance of the enamel matrix in Figures 5A and 5B, may, at least in part, be due to the different degree of maceration between these two samples. Some of the globular masses in Figure 5A may, according to these authors, derive from the reaggregation of extracted material. The preservation of organelles obtained in our study is, in part, discussed in the answer to the previous question. Also, their general appearance is similar to that obtained by high-resolution SEM in other cell types and by transmission electron microscopy in ameloblasts as well as in other cell types.

Reviewer IV: Please clarify the new findings that the authors want to present in this paper!

Authors: Although the present data are confirmatory of previous observations, they further extend these observations, clearly establish the extensiveness and three-dimensional organization of the Golgi apparatus in ameloblasts, particularly from the maturation stage, and show a possible correlation of the smooth tubular network with endosomal elements. The data are also discussed in terms of recent findings on the formation and degradation of enamel proteins, and on the nature of the endosomal compartment.

D.M. Lyaruu: What, according to you is the rationale (or function) of enamel matrix secretion by the maturation stage ameloblasts if the matrix having the same immunocytochemical reaction already deposited into the enamel during the secretory stage of amelogenesis has to be degraded and removed from the tissue during the maturation stage of amelogenesis?

Authors: The role of continued enamel protein release during the maturation stage is not clear at this time. It is possible that it may represent a residual expression of the intense secretory activity during the secretory stage. Alternatively, some "typical" or "novel" enamel protein (including enzymes), may be released to complete the maturation process.

Y. Higashi: Could the authors be so kind to tell us of any morphological evidence for differentiating the secretory granules form lysosomal/endosomal elements of the ameloblast in this SEM study?

Authors: Indeed, it is difficult to distinguish secretory granules from some lysosomal elements in SEM preparations. We have, therefore, utilized throughout the text the expression "secretory-like granules" to indicate probable secretory granules. The criteria used to distinguish, albeit arbitrarily, between secretory granules and lysosomes are size and shape, and their general distribution and frequency as determined from various published transmission electron microscope studies. Even in the latter case, unless cytochemical labeling is performed, it is not always possible to distinguish secretory granules from small, acid hydrolase-positive, granules (lysosomes?) present in Tomes' process, for instance. This is not the case, however, for the secondary lysosomes found in the supranuclear compartment of ameloblasts, which are generally larger in size.