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SCANNING ELECTRON MICROSCOPY OF THE FORMING ENAMEL OF RAT INCISOR: INFLUENCE OF FIXATIVE AND TREATMENTS INTERACTING WITH THE ORGANIC MATRIX

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Abstract

The forming enamel of rat incisor was either treated before fixation with one of these five treatments: ultrasonication, NaOCl, chloroform/methanol, NaOH or tween 20; or the incisors were first fixed and then submitted to one of these treatments. The structure of the forming enamel, compared with sections fixed with aldehyde and submitted to osmic maceration, depends on the way the samples were treated and on the fixative used. An amorphous material, removed by osmium tetroxide but not by aldehyde solution was seen to be located between fiber-like structures. In the region of inner enamel secretion the thickness of interrod septa was fairly constant, whereas in the region of outer enamel secretion, transverse septa of interrod enamel gradually became thinner and finally disappeared. The longitudinal septa did not display such changes. Longitudinal grooves and ridges were seen at the end of enamel completion. These longitudinal septa finally merged and formed a flat surface.

KEY WORDS: Forming enamel, rat incisor, interrod and rod enamel mineralization, ultrasonication, sodium hypochlorite, sodium hydroxide, tween 20, chloroform/methanol-aldehyde fixation, osmium tetroxide.

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Introduction

The structure of dental enamel of many species has been observed by several authors (for a review see: Sasaki et al., 1990). For these studies, the scanning electron microscope (SEM) is a very powerful tool that is widely used (for a review see: Boyde et al., 1988). The formation and development of enamel has been extensively studied in the rat incisor which constitutes a very suitable model because, in a continuously growing and erupting tooth, the complete sequence of enamel secretion and maturation may be observed from the forming apex of the tooth to the erupting zone (for reviews, see: Leblond and Warshawsky 1979, Warshawsky 1979). A few reports give information on the structure of the forming and maturing enamel (observed with the SEM) of the rat or the mouse incisor (Boyde and Reith 1976, 1977; Risnes 1979a; Skobe 1980; Skobe et al., 1985; Sasaki et al., 1987; Ashrafi et al., 1988). Most of these authors used material fixed by perfusion or immersed in fixative solutions such as glutaraldehyde, glutaraldehyde-paraformaldehyde mixtures or osmium tetroxide. Therefore, the structures observed may be influenced by such treatment. In this report we investigated the appearance of the surface of forming and maturing enamel of rat incisor using various treatments and fixation methods. These treatments, known to remove mechanically or chemically either cell remnants or partially or totally the organic matrix, were aimed at unmasking the native structure of the forming enamel.

Materials and Methods

Forty Sprague-Dawley rats 200-250 grams in weight were killed by an overdose of intraperitoneally injected chloral solution. The mandibles were dissected and the two lower incisors removed. Then, 40 incisors were immersed in the following fixative solutions: 20 teeth were fixed for two hours at 4°C in a mixture consisting of 2.5% glutaraldehyde and 2.0% paraformaldehyde, in 0.1 M sodium cacodylate buffer, pH 7.2 (Karnovsky 1965) followed by 2.0% osmium tetroxide in the same

buffer for one hour at room temperature (**group I**). The remaining 20 incisors were immersed in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer, for two hours at room temperature (**group II**). Four teeth in each of these groups were rinsed in the buffer then treated in the following way: either (**1**) ultrasonicated in 10 ml phosphate buffer, pH 7.4 at room temperature for 10 minutes to mechanically remove cell remnants; or (**2**) submitted to 10 ml NaOCl 50% for 2 hours at room temperature, in order to chemically remove the organic material; or (**3**) submitted to the action of a chloroform/methanol (2:1 v/v) mixture (10 ml) at room temperature for 2 hours to remove lipids; or (**4**) treated in 10 ml 6N NaOH at 60°C for 20 minutes (this maceration method has been shown to remove the cellular and intercellular elements from specimens that may obscure the details of interest, see: Takahashi-Iwanaga and Fujita, 1986); or (**5**) the incisors were immersed in 10 ml 1% tween 20 in phosphate buffer for 2 hours. Such surface agents with solubilizing effects are able to remove part of the extracellular materials (Shimada et al., 1981).

Another series of 40 incisors was first submitted to one of the treatments 1 to 5 (see above) and then, either fixed as in group I with a glutaraldehyde-paraformaldehyde solution, followed by osmium tetroxide post-fixation (**group III** - 20 teeth) or as in group II fixed only with 2% OsO_4 (**group IV** - 20 teeth).

This material was compared with the incisors obtained from two rats perfused through the heart with the Karnovsky solution for 10 minutes. The mandibles were immersed for 1 hour in the fixative solution, sectioned with a sawing machine (Accutom-Struers, Copenhagen, Denmark). The 1-2 mm thick sections, including the bone and the incisor, were then submitted to the osmic maceration procedure developed by Tanaka and Mitsushima (1984): 0.1% osmium tetroxide for 72 hours at 20°C (**group V**).

All the incisors and the transverse sections of the rat mandibles were further dehydrated in graded ethanol, critical point dried, glued to aluminium stubs, sputter-coated with gold-palladium and examined in a JEOL JSM 35C SEM operating at 20 kV.

Results

Appearance of the forming enamel and effects of the treatments

It was clear from our results that initial fixation reduced or even suppressed the effects of the various treatments. Treatments carried out on group III and IV incisors gave the best results, whereas partial layers or groups of persisting cells or remnants of Tomes' processes obscured the observation of the forming enamel in groups I and II. The forming enamel surface was clearly observed after sonication or sodium hypochlorite or sodium hydroxide treatments, but to a lesser extent when chloroform-methanol or tween 20 treatments were used before aldehyde fixation (Figs. 1 and 2).

Fig. 1. Sodium hydroxide treated rat incisor before fixation (group III). Rows of forming rods are clearly seen. Bar = 100 μm.

Fig. 2. Rat incisor fixed (group I) and then treated with tween 20. Remnants of cells are seen on the enamel surface (asterisks). Bar = 1 mm.

Fig. 3. Forming enamel of rat incisor after perfusion through the heart with aldehyde and osmium tetroxide maceration (group V). Bar = 1 μm.

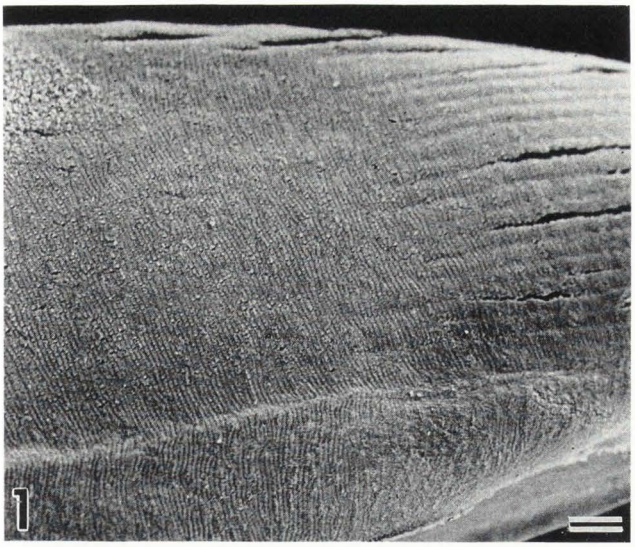
Fig. 4. Same fixation procedure as in Figure 3. At this magnification, the deepest part of the alveoli appears irregular. Holes result from projections or villi of the Tomes' processes. The external part of the interrod septa seems to be constituted of rounded granular material (arrowhead). Bar = 1 μm.

Fig. 5. Treatment with tween 20 followed by aldehyde fixation (group III). The septa appear swollen. Bar = 1 μm.

Fig. 6. Treatment with tween 20 followed by osmium tetroxide fixation (group IV) enables observation of a network of fibers and granules. Spaces between this material are empty. Bar = 1 μm.

The thickness of the interrod septa in the forming enamel varied according to the method used and to the treatment performed. In the zone of inner enamel formation longitudinal septa, parallel to the long axis of the incisor, were seen to vary between 0.5 and 1 μm in thickness on the surfaces of group V incisors, whereas their transverse septa, at right angles to the long axis of the teeth was about 0.2 μm thick (Figs. 3 and 4). Treatments with tween 20 before fixation seemed to induce some swelling of these septa (1.6 and 0.7 μm, respectively) (Figs. 5 and 6), whereas sodium hypochlorite or sodium hydroxide reduced their thickness to thin leaflets (Figs. 7 and 8). From a posterior to a more anterior area in the forming zone, the transverse septa became progressively thinner.

At higher magnification, pits which contained the Tomes' processes before osmium maceration (group V), looked relatively smooth and dense (Fig. 4). We could see that the surface of these cavities consisted of granular material, each granule was about 90 nm in diameter. Holes in the deepest part of these pits were also observed, supporting the view that the Tomes' processes have digitations or protuberances arising from their distal surface. The septa of the interrod enamel were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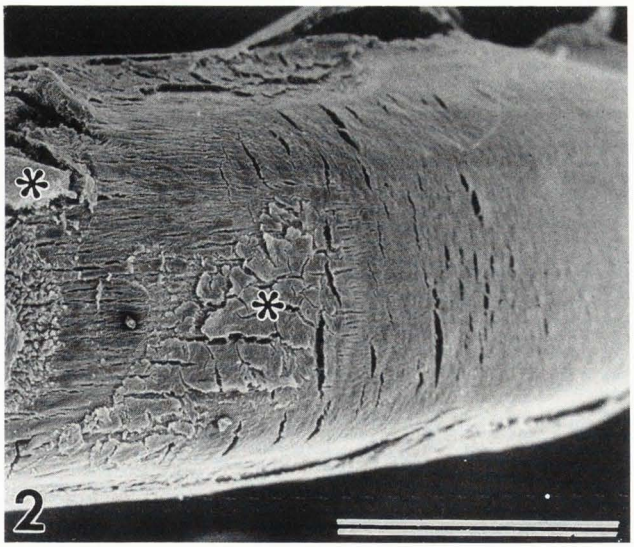
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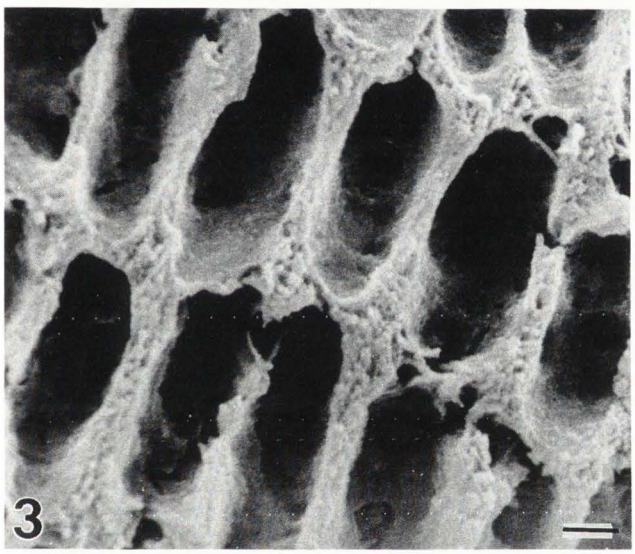
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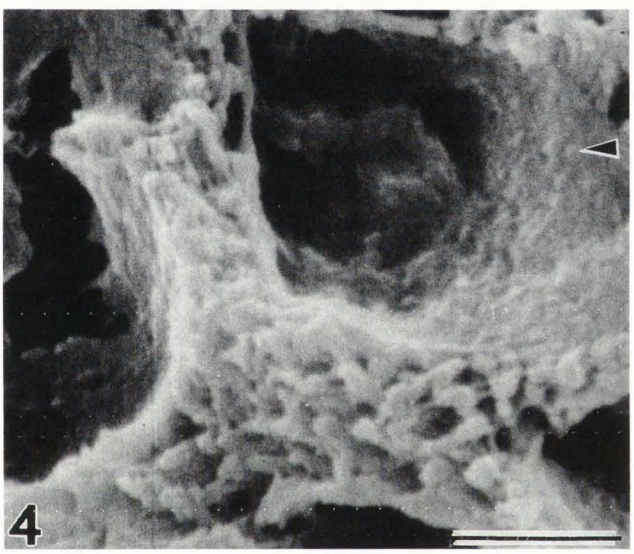
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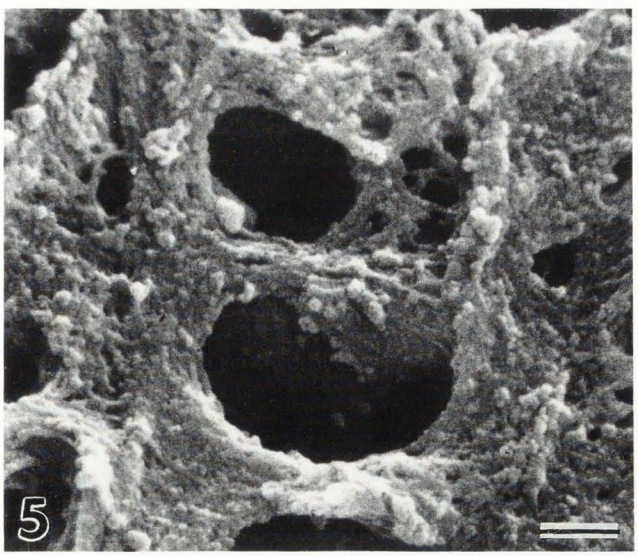
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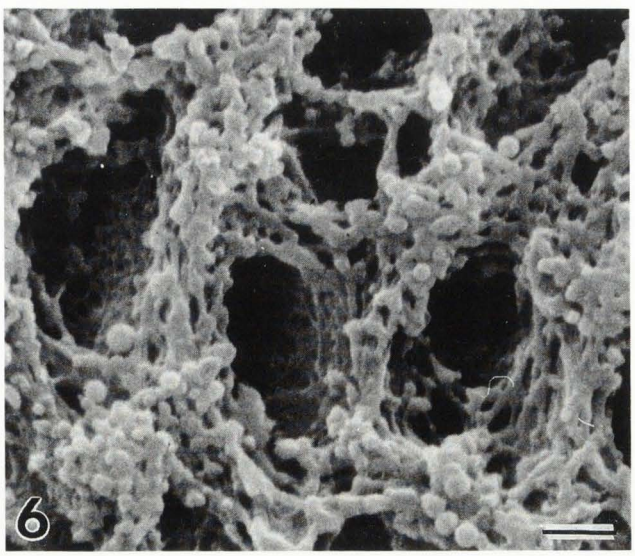
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composed of dense material peripherally and were porous centrally. The presence of fibrous structures, 130 nm thick and well individualized but connected with thin bridges, gave this material a rough appearance. After ultrasonication, the thickness of the septa was reduced, and they seemed to be made up of a 125 nm thick sheet of fibers (Figs. 9 and 10). This suggests that during ultrasonication, part of the most recently deposited material was lost and only the older one or the mineralizing material resisted such treatment. Sodium hypochlorite and sodium hydroxide significantly reduced the forming interrod enamel where fibrous substructures were still distinct on smooth surfaces (Figs. 7 and 8). Less material was removed with sodium hydroxide than with sodium hypochlorite. Chloroform-methanol treatment only cleaned the spaces occupied by the Tomes' processes. Treatment with tween 20 containing solutions followed by aldehyde fixation deprived surfaces of membrane remnants, but swollen septa could be noted, consisting of granules and fibers and a coating material which obscured this organization (Fig. 5). On the other hand tween 20 treatment followed by osmium tetroxide fixation removed the coating material and left a swollen network of rounded granules, 300 nm in diameter and an network of 200 nm thick fibers (Figs. 6 and 11). Spaces between this material were empty. None of these appearances were visible or were blurred when aldehyde fixation was carried out before the various treatments (groups I and II).

Changes in the appearance from the forming to the maturing enamel

From the forming end of the mandibular incisors towards the maturing enamel, separate zones were observed after various treatments (Fig. 12). Near the apical loop of non-treated teeth, a region with differentiating and presecretory ameloblasts was present. This portion was totally or partially lost after delipidation, deprotinization or tween treatments. Therefore, the apical zone observed on our treated preparations, started with the formation of the first layer of mineralized dentine. The zone of inner formation could be seen in the form of more or less regular transverse rows of oval or square boxes (Fig. 10). These pits had contained the Tomes' processes. They were defined by interrod enamel. The length of this portion was about 4 mm. This configuration was most distinct in the center of the labial portion of the teeth, whereas in the lateral portion the organization into rows was not so clear. With the increasing thickness of the forming enamel, it was obvious that the transverse septa of interrod enamel became thinner than the longitudinal septa (Fig. 13). A 0.6 mm long C-shaped band constituted an intermediary zone. The pits containing Tomes' processes became elongated.

Fig. 7. Sodium hypochlorite removed part of the material of the interrod septa and left clean leaflets of mineralized material (fixation group III. Bar = 10 μm.

Fig. 8. A similar appearance as seen in Figure 7 is observed after treatment with sodium hydroxide (fixation group III). Bar = 10 μm.

Fig. 9. Sonication followed by fixation with aldehyde solution (group III) partially removes interrod components but allows clear visualization of fiber-like structures. Bar = 10 μm.

Fig. 10. Same treatment as in Figure 9 observed at a higher magnification. Bundles of fibers seem to be the main structure of the interrod enamel. Bar = 1 μm.

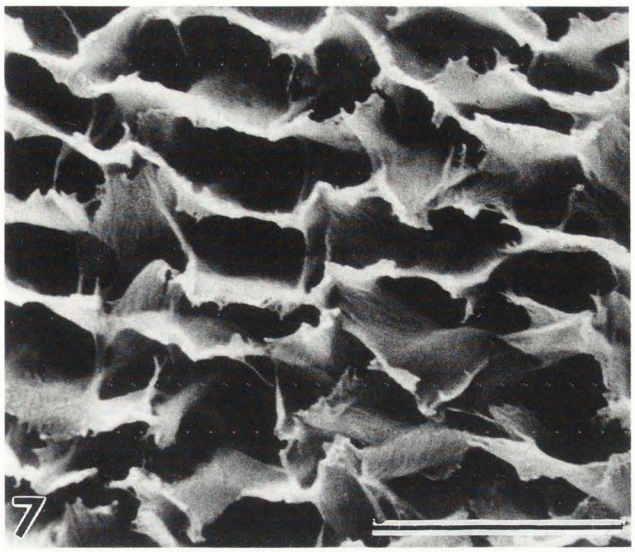
Fig. 11. After tween treatment, followed by osmium tetroxide fixation (group IV), the forming matrix appears as granules (arrowhead) interconnected by thick tubules. The comparison between Figures 10 and 11 demonstrates the importance of the treatment and the fixative procedure. Bar= 1 μm.

Fig. 12. Low magnification of a rat incisor submitted to the action of sodium hypochlorite then fixed with aldehyde solution (group III). From the forming apex, on the left of the figure to the early maturing enamel, on the right, the appearance of the surface varies. Bar = 1 mm.

Another transition could be seen in a 0.35 mm band where only a longitudinal organization was clearly seen. This leads to the formation of longitudinal ridges, 5 to 10 μm thick, separated by 40-55 μm wide grooves. This 0.8 mm long C-shaped band contained longitudinal septa which were connected at some places, thus forming a network (Fig. 14). It preceded a final distinct band where the 10 μm thick parallel ridges became progressively closer, the grooves being about 10 μm wide (Fig. 15). Finally, these bands and grooves seemed to merge (Fig. 16), and the surface of the maturing enamel became smooth. This last forming area was 0.6 mm long. On the whole, the area where we saw the enamel growing to completion and to maturation was about 4 mm long.

Discussion

The present observations address two questions. Firstly, what is the real structure of the forming enamel, and secondly, what is the reason for the variation in appearance between the forming part at its earlier stages and the area where enamel starts to mature?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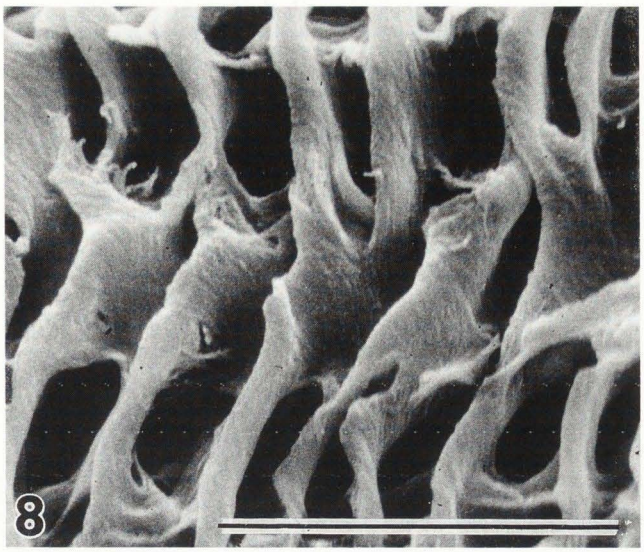
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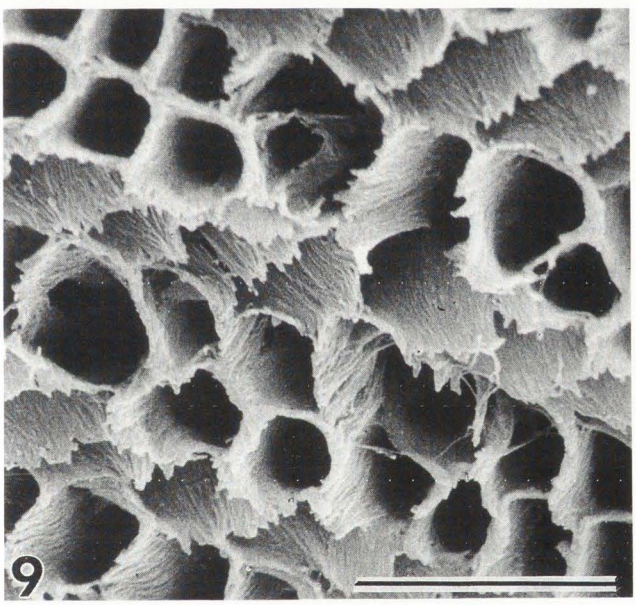
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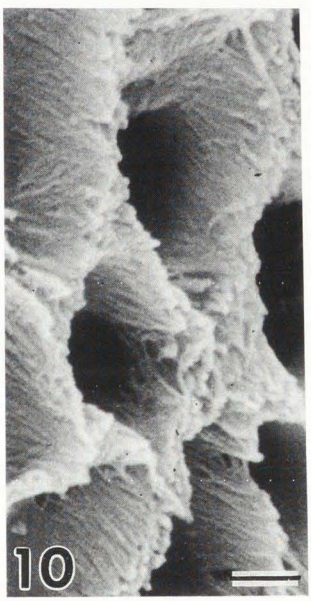
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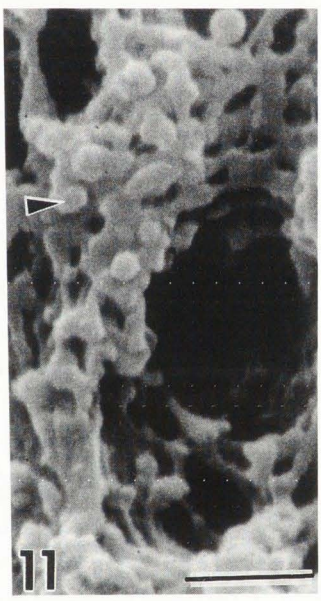
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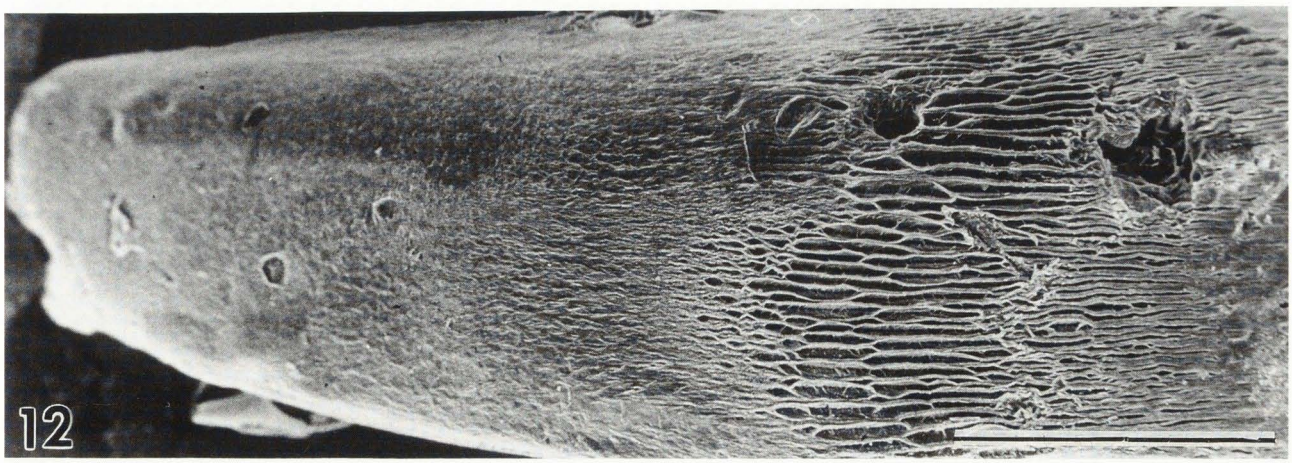
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Organization of the forming enamel

With the transmission electron microscope, the forming enamel was described as fibre-like structures interconnected with thin fibrils (Travis and Glimcher 1964, Sasaki et al., 1990). Such 12-18 nm wide fibrous structures were separated by spaces whose width varied between 24 and 48 nm. These accurate measurements obtained after aldehyde fixation (Goldberg and Septier 1986) as well as after anhydrous fixations using rapid freezing and freeze substitution (Goldberg and Escaig 1982, Sasaki et al., 1990) did not agree with our measurements on SEM pictures. On these, the forming enamel either appeared as 90 nm diameter granules or as 130 nm thick fibrous material. That may be explained by the fact that the fibers observed with the SEM are not individual fibers but bundles of fibers containing a certain amount of collapsed fibers, the whole lot still keeping a fibre-like appearance. It follows from our results that: (1) only treatments performed before fixation allow visualization of the substructure of the forming enamel, and (2) the statement that osmium tetroxide is a better fixative for SEM than aldehyde solutions is confirmed. Therefore, we must be cautious and take into consideration the way the tissue was treated before we draw any conclusions on the real organization of the forming enamel. Combining the results obtained after these various treatments, we come to the conclusion that the native structure of the forming enamel consists of fiber-like structures embedded in an amorphous material, this later being removed by osmium tetroxide maceration or fixation. Reaggregations of partially extracted material gave rise to granules and fiber-like structures or network. Tween 20 was seen to induce some swelling of the septa. Part of this material disappears after treatment with solutions destroying matrix components, such as sodium hypochlorite or sodium hydroxide, leaving thinner septa of material which is probably already mineralized. In this respect, we have no information on how the different treatments affected precipitation of calcium salt during the treatment and the subsequent dehydration but obviously this phenomenon influences the appearance of the surface of the forming enamel. We may conclude from this series of investigations that the appearance of the forming enamel is directly related to the preparational treatment of the samples. It is clear that intracardiac fixation followed by osmium tetroxide maceration gives the most reliable appearance, whereas each treatment used in this report removed some enamel matrix component. The effects of such treatments were visible only when they were performed before fixation. Combined together, the appearance observed after using such subtractive methods allow morphological approach of the native structure of the forming enamel.

Fig. 13. During the formation of the outer prismatic enamel, there is a decrease in the formation of transverse septa. Only longitudinal septa are laid down. Bar = 10 μm.

Fig. 14. Longitudinal ridges and grooves seen here after sonication and aldehyde fixation (group III). Bar = 100 μm.

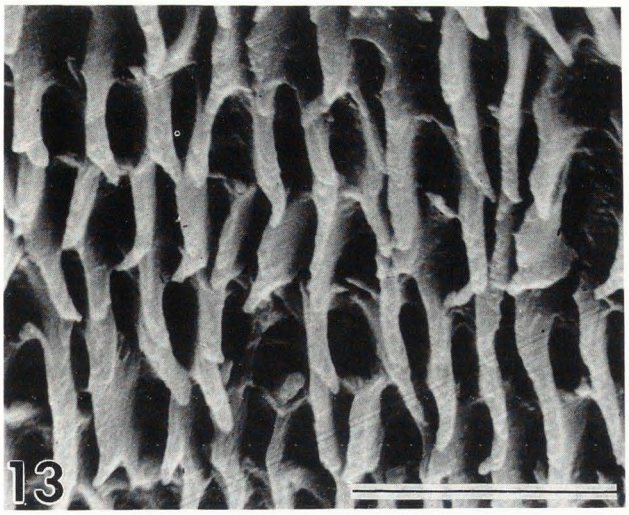
Fig. 15. Spaces between the ridges become narrower than in Figure 14. Sonication and aldehyde fixation (group III). Bar = 10 μm.

Fig. 16. The longitudinal bands converge, merge and finally contribute to the formation of a smooth enamel surface. Bar = 100 μm.

Variations between the forming end and the maturation zone

Since long, variations have been reported between the forming end of the rat incisor and the erupted portion (Warshawsky and Smith 1974). Robinson et al., 1979 divided the rat incisor into a forming zone followed by a transitional zone and a maturing zone. A more precise picture emerges from the figure drawn by Leblond and Warshawsky (1979) and by Warshawsky (1979), which recognized a zone of presecretion, followed by a zone of enamel secretion of immature enamel, subdivided into the region of initial enamel secretion, the region of inner enamel secretion, the region of outer enamel secretion, and the region of final enamel secretion. The maturation zone itself is divided into a modulation and a pigmentation zone. The erupted portion of the teeth constituted the last portion of the tooth. From the apex to the point where maturation starts, according to these measures, the total length is 7.3 mm. If the presecretory zone is not taken into account, and pointing out that the incisor is a curved structure, the 4 mm found with the SEM fits rather well with the 5.2 mm calculated by Warshawsky (1979) from light and transmission electron microscope observations.

The main finding of our report is that eventually transverse septa are reduced and disappear. Further longitudinal crests and grooves gradually come to confluence in the region of final enamel secretion. This was not seen on thin sections because they are usually cut in a transverse plane to the long axis of the tooth, therefore longitudinal interprismatic septa are always present between Tomes' processes. The series of investigations reported here allow us to conclude that induced artifacts can be observed in the organization of the forming enamel. This latter one appears either in the form of fiber-like structures, or as granules, or as networks, which are questionable. However, grooves or fissures were never seen in the forming zone. Alignments of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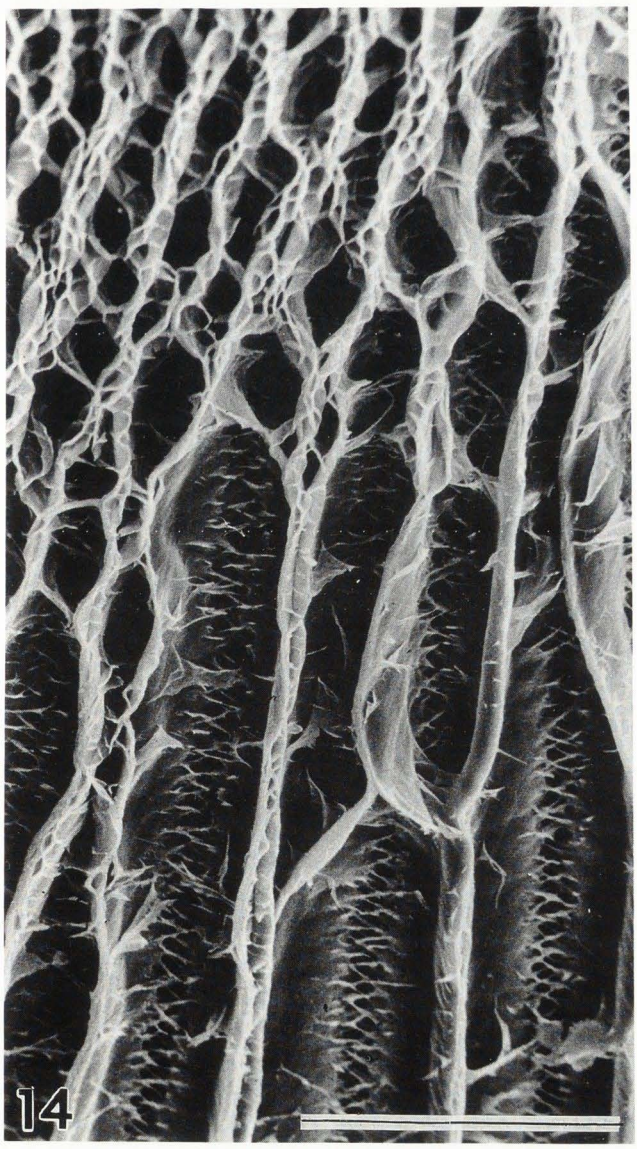
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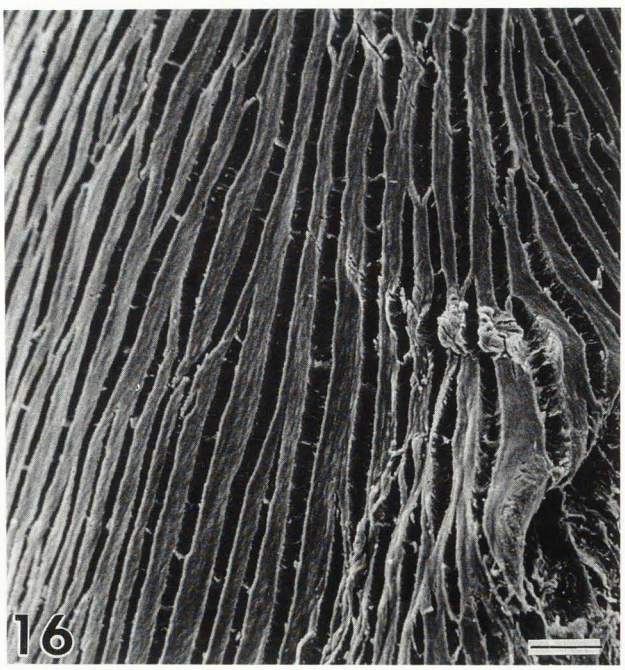
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pits and the network of interrod enamel never showed artifactual distortions. Therefore, it is very unlikely that the configuration seen in the transition and maturation zone is an artefact caused by critical point drying which occurs only in this area. We have to admit that the secretion of interrod material, which at the onset of enamel formation occurs all around the Tomes' processes, is gradually restricted to two lateral surfaces only. This is the reason for the reduced thickness of the transverse septa and their complete disappearance, a fact which has now to be reinvestigated on longitudinal thin sections with the transmission electron microscope. In this respect, it is well known that during outer enamel formation the Tomes' processes become nearly horizontal (Kallenbach 1973, Warshawsky and Smith 1974, Skobe 1976). Therefore, the changes in the cell shape seem to play a key role in the formation of longitudinal crests and grooves. This, as well as the evidence of C-shaped zones or bands, first described by Smith and Warshawsky (1976), result from the fact that ameloblasts move faster than the tooth erupts (Risnes 1979b). The rate of migration of the cells was found to be 631 μm/day, whereas the eruption is usually given as 400 μm/day (Smith and Warshawsky 1975). Thus, it may be concluded that the appearance of the surface in the region of final enamel secretion differs substantially from the region of inner and outer enamel secretion, and is influenced both by the eventual changes in cell shape and by erupting forces.

Much is known about the mode of enamel formation at early stages. Our knowledge is also consistent with respect to enamel maturation. Obviously, there is a gap in our knowledge and more investigation has to be carried out on the transition area seen between the zone of outer enamel secretion and early maturation zone. Whether or not the facts reported here are unique and only related to the rat incisor development or if this is a more general phenomenon during amelogenesis is a point which needs further investigation.

Acknowledgements

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Discussion with Reviewers

S. Lindskog: Was there a specimen of developing enamel in its native stage to compare with ?

Authors: To the best of our knowledge, it is impossible to observe developing enamel in its native state with the SEM, without dehydration. As soon as the samples are dehydrated, they are no more in a native state. Shrinkage occurs, which strongly interferes with the appearance of the forming enamel. Rapidly frozen and freeze-substituted material, observed on replica with the TEM may constitute the best methodology (Sasaki, Goldberg, et al., 1990) for such an approach, although freezing may introduce, by itself, its own artefact. This is the reason why in this report our aim was to compare the forming enamel submitted to various treatments before or after fixation. We are fully aware that each method carries, by itself, its own denaturation of the native structure.

S. Risnes: Which of the presented micrographs do you think represent the condition which morphologically comes closest to the true nature of the enamel matrix after removal of the ameloblasts?

Authors: Previous investigations support the concept that the forming enamel is constituted by tubular structures, thin bridges giving a ladder-like appearance, and an amorphous coat (Travis and Glimcher, 1964; Goldberg and Septier, 1986; Sasaki, Goldberg, et al., 1990). Perfusion through the heart with aldehyde followed by osmium tetroxide maceration gave the appearance which was the closest to what was observed with the TEM (Figs. 3 and 4), therefore this may be the closest to the true nature of the forming enamel. However, this does not imply that all enamel components are present keeping their exact size without distention, but, on the contrary, are partly or totally removed by the method used.

S. Risnes: When comparing Figures 7 and 8 with Figures 9 and 10, which components do you think have been removed and which components remain after these treatments?

Authors: Sodium hypochlorite as well as sodium hydroxide remove large portions of the organic matrix. Therefore, we observe on Figures 7 and 8 the mineralized part of the forming enamel, as well as some matrix remnants which probably are still present after treatments lasting 2 hours for sodium hypochlorite and 20 minutes for sodium hydroxide. These thin bended leaflets are in strong contrast with the thicker material observed after sonication. This treatment is aimed to mechanically remove cell remnants, but it also eliminates the most recently secreted enamel matrix, not yet organized in the form of fibers. These later fibers obviously display better resistance to such treatments.

J.H.M. Wöltgens: In the "Results" section, it is mentioned that "Treatments carried out on group III and IV gave the best results" without giving the criteria used to evaluate the effect of the various treatments on the surface morphology!

Authors: Our aim was to observe the surface of the developing enamel. When treatments were carried on fixed enamel, persisting cells, remnants of Tomes' processes, and a coating glue obscured the observation. Better visualization of the forming enamel was obtained when the samples were submitted to treatments and then fixed either with aldehyde and osmium tetroxide or only with osmium tetroxide. In that sense groups III and IV gave better results than groups I and II. Except tween 20 which induced swelling and globular alterations of the forming enamel, each treatment performed on groups III to V revealed mostly all or some matrix and/or mineral component of the developing enamel. The comparison of all these results permits a morphological approach of the native structure. It is also clear that each method is questionable and carries its own artifacts.