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MULTIPLANE SECTIONING AND SCANNING ELECTRON MICROSCOPY AS A METHOD FOR STUDYING THE THREE-DIMENSIONAL STRUCTURE OF MATURE DENTAL ENAMEL

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Abstract

A method is described where teeth are sectioned/ground along at least two planes, etched, and viewed at various angles in the SEM in order to study the three-dimensional structure of enamel. This multiplane sectioning-scanning electron microscopy (MPS-SEM) method has been applied to the study of rat and human enamel. The method demonstrates in a direct way the complex three-dimensional structure of rat incisor enamel; the path of prisms and the distribution of interprismatic substance. The different appearance of alternate prism rows as seen in the longitudinal plane is seen to be due to prisms in alternate rows being inclined to different degrees in the transverse plane. In human enamel, the method reveals the three-dimensional nature of Retzius lines. In tangential planes cut at right angles to the prisms, Retzius lines are identified on the basis of an altered size, shape or etching pattern of prisms. Considerable variation in etching pattern within the same tangential plane was observed. Enamel tufts could be observed simultaneously in tangential and longitudinal planes, giving information about their three-dimensional extent and their relationship to Hunter-Schreger bands and to prisms and interprismatic substance. The main advantage of the method is that deduction of three-dimensional structure from the appearance of the structure in one plane is aided by the appearance of the structure in the adjoining plane.

KEY WORDS: Dental enamel, prisms, Retzius lines, tufts, acid-etching, multiplane sectioning, three-dimensional structure, scanning electron microscopy

Introduction

Knowledge of the three-dimensional structure of a cell, a tissue or an organ is important for understanding its morphogenesis, functions and reactions. With the light microscope and the transmission electron microscope, the three-dimensional structure of a biological specimen can be disclosed by observing serial sections and subsequent graphical or model reconstruction (Gaunt, 1971; Gaunt and Gaunt, 1978; Ware and LoPresti, 1975). However, this is an elaborate and time-consuming process, although progress has been made with the introduction of computer-based image analyzing and processing techniques (Ware and LoPresti, 1975; Moens and Moens, 1981).

Dental enamel, which is the hardest tissue of the body, has a complex three-dimensional structure. The structural richness of dental enamel is related to the spatial arrangement of the closely packed hydroxyapatite crystals. These are not arranged at random; in mammals, the crystals are organized into a pattern of prisms (rods) and interprismatic (inter-rod) substance. Basically, the prisms are discrete, rod-like entities running from the dentine to the enamel surface, while the interprismatic substance constitutes a continuum in between the prisms. The hydroxyapatite crystals are differently oriented in prisms and interprismatic substance, and this is the sole basis for a distinction between the two (Boyde, 1964; Warshawsky et al., 1981).

Crystal orientation is governed by the enamel-producing cells, the ameloblasts; each prism is formed in relation to one and the same ameloblast throughout amelogenesis, while the interprismatic substance is formed at the border region between two or more ameloblasts (Warshawsky et al., 1981; Nanci and Warshawsky, 1984).

Due to its hardness, mature dental enamel is not suitable for conventional

serial sectioning. The scanning electron microscope, detecting signals from the surface of bulk specimens and providing a great depth of focus and high resolution, offers, in combination with multiplane sectioning of teeth, a unique possibility for direct visualization of certain aspects of the three-dimensional structure of mature dental enamel.

Materials and Methods

Due to its high degree of mineralization, dental enamel is a very stable tissue. The structure of mature enamel is, therefore, largely unaffected by the post-extraction handling of the tooth, whether it is fixed or not, and whether it is stored wet or dry. When stored wet, the pH of the liquid should be checked in order to avoid demineralization. Teeth which had been stored wet, for instance in 70% alcohol or in water, were found to be somewhat less liable to cracking during sectioning and grinding compared with dried teeth. However, dried teeth were fully acceptable as material for the kind of work reported here, i.e. the study of mature enamel structure.

In order to demonstrate three-dimensional aspects of internal enamel structure in the scanning electron microscope, each tooth was sectioned and ground along at least two planes. First, the position and direction of the planes were carefully planned. After initial sectioning (Risnes, 1981) the specimen was fixed to a small, cylindrical metal stub by a drop of cyanoacrylate glue, which sets fast and gives a strong bond. The specimen could now be further sectioned and ground in a specially designed apparatus (Risnes, 1985a). In order to reveal the enamel structure, the sectioned and ground surfaces had to be etched with acid. Concerning the choice of etching agent, a great number of both strong and weak acids would be acceptable. However, since the etching pattern varies with type of acid, acid strength, etching time, and stirring rate (Poole and Johnson, 1967; Fosse, 1968; Johnson et al., 1971; Silverstone et al., 1975; Tyler, 1977; Boyde et al., 1978; Dennison and Craig, 1978; Dijkman et al., 1983), it was necessary to standardize the procedure in order to make interpretation more reliable. The procedure employed throughout this study was as follows: The specimen was held by a pair of tweezers, dipped and stirred by hand in a jar containing 1.3% nitric acid (pH 0.87) for 3 periods of 5 seconds interrupted by washing under running tap water. Nitric acid was chosen because it had given adequate and reproducible results in earlier studies (Fosse, 1968; Fosse et al., 1973). The strength 1.3% was chosen because an action time of 3 times 5 seconds would remove a surface layer of about 5 µm (Fosse, 1968), which is enough to eliminate the superficial enamel layer that may have been structurally damaged during the grinding/polishing procedures (Boyde and Stewart, 1962). The interrupted etching procedure preserves surface planarity (Fosse, 1968).

Following etching, the specimen was rinsed under running tap water and air-dried. If necessary, silver paste was used to reduce charging by painting the specimen in strategic regions (Thornton, 1968).

Although enamel structure can be observed in the scanning electron microscope without coating (Risnes and Stölen, 1981), a thin layer of metal will effectively reduce charging and optimize the resultant image, especially at accelerating voltages above 3 kV. The specimens were sputter-coated with a 40 nm layer of gold or gold-palladium.

Figs. 1 - 16. Abbreviations: D = dentine, E = enamel, ET = enamel tuft, IPS = interprismatic substance, LP = longitudinal plane, P = enamel prism, S = surface of enamel, TP = transverse plane, TaP = tangential plane, e-e = edge between sectioned planes, ie = inner enamel zone, oe = outer enamel zone.

Fig. 1. Mesial half of lower left rat incisor sectioned along three planes. Labial aspect viewed from behind and somewhat distally. Bar = 100 µm.

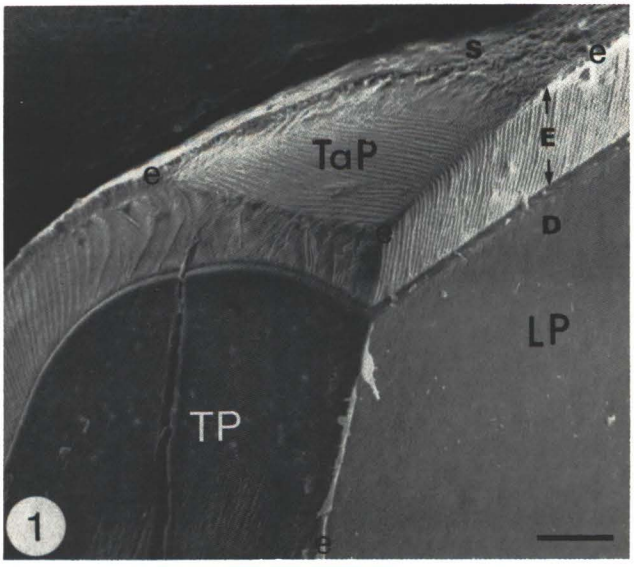
Fig. 2. Higher magnification of area in Fig. 1 showing the appearance of the inner enamel zone in three different planes. Prisms of alternate, transverse rows are inclined in opposite directions (small arrows), but not to the same extent relative to the longitudinal plane (large arrows). Bar = 50 µm.

Fig. 3. Higher magnification of area in Fig. 1 where the transverse plane meets the tangential plane. Prisms crossing in the inner enamel zone become parallel and increasingly incisally inclined in the outer enamel zone. Bar = 50 µm.

Fig. 4. Prisms of alternate rows (unlabelled arrows) show different appearances in the longitudinal plane depending on the transverse inclination of prisms. The interprismatic substance crystals are oriented about 90° relative to the prism direction. Bar = 10 µm.

Fig. 5. Mesial half of rat upper left incisor sectioned longitudinally and transversely. Labial aspect viewed from the front and somewhat distally. Bar = 100 µm.

Fig. 6. Higher magnification of Fig. 5. The interprismatic substance is organized into radially directed sheets. The incisal inclination of prisms in the inner enamel zone diminishes toward the outer enamel zone (unlabelled arrows). Bar = 10 µm.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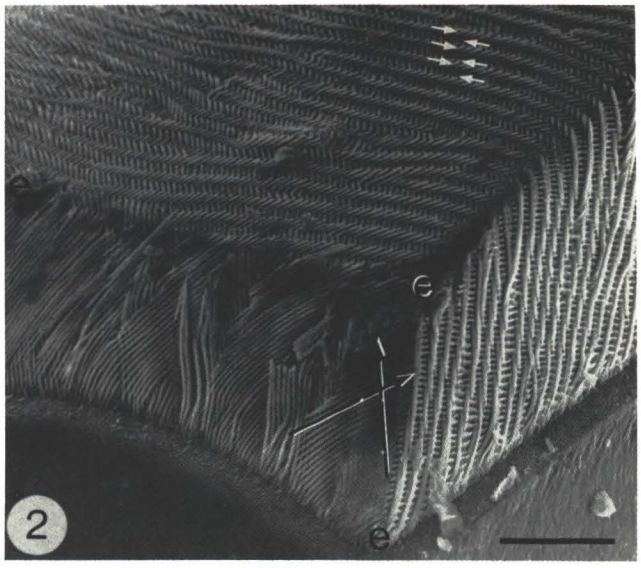
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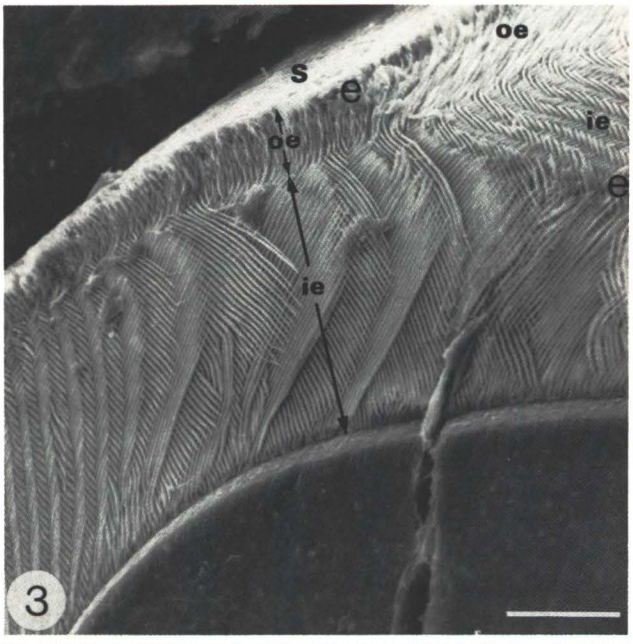
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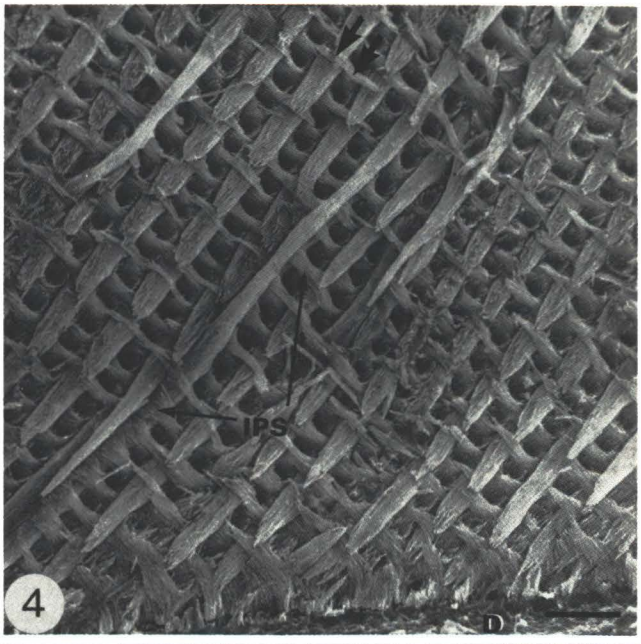
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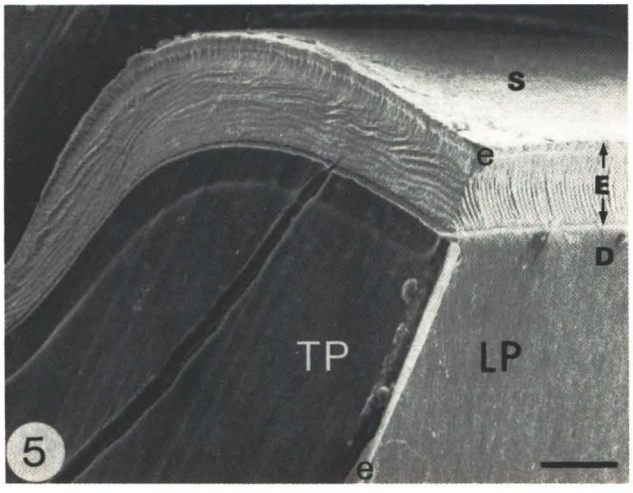
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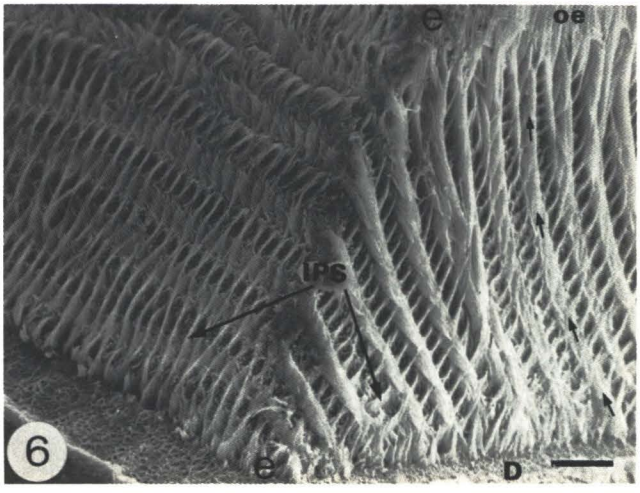
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In order to be able to view different aspects of the same enamel specimen in the SEM, a holder was constructed which allowed perpendicular viewing of all specimen aspects deviating as much as 135° from the top surface (Risnes, 1982). The JEOL 50A was operated at 15-20 kV.

The multiplane sectioning-scanning electron microscopy (MPS-SEM) method outlined here has been applied to the study of mature rat and human dental enamel.

Results

Rat enamel

The constantly growing rat incisor is covered with enamel on its labial aspect (Figs. 1 and 5). The enamel has a thickness of about 100 µm in the upper incisor and about 140 µm in the lower incisor. The prisms are organized into a striking pattern; prisms of alternate, single-layered, incisally inclined, transverse rows cross each other at about 90°. In the outer 20-30 µm thick zone all prisms become parallel and approach the surface at an acute angle of about 25°. This complex, yet highly regular uniserial lamellar prism pattern is readily demonstrated in an incisor which has been sectioned/ground along three different planes; longitudinal, oblique transverse, and tangential (Figs. 1-3). From a specimen prepared in this way the course of the prisms within the three-dimensional enamel cap can be inferred, although it is not possible to directly follow the complete path of any particular prism.

When a rat incisor is bisected longitudinally in mesial and distal halves, alternate prism rows usually have different appearances in the resulting longitudinal plane; in one set of rows prisms are cut more transversely than in the other set, in which the prisms are cut more longitudinally (Figs. 2,4,6). The explanation can be inferred from the adjoining oblique transverse plane where prisms of alternate rows are seen to be inclined at different angles relative to a longitudinal plane perpendicular to the enamel-dentine junction (Fig. 2).

The interprismatic substance separating the prisms within the same row is easily identified in longitudinal sections (Fig. 4). The crystals comprising the interprismatic substance are oriented approximately at right angles to the direction of the prisms. In an oblique transverse section, which is cut nearly at right angles to the prisms and consequently nearly parallel with the interprismatic substance crystals, a system of radially directed sheets of interprismatic substance running from the dentine-enamel junction toward the enamel surface is clearly visible (Fig. 6).

In the oblique transverse plane shown in Figure 6, the cross-cut prisms in the outer part of the inner enamel zone appears larger and more pointed than prisms closer to the dentine. By referring to the adjoining longitudinal plane it can be seen that this phenomenon is caused by a diminished incisal inclination of the prisms in the outer part of the inner enamel zone.

Human enamel

Retzius lines. With the method outlined here it is very easy to show directly that Retzius lines are continuous across the edge where two planes meet (Fig. 7), revealing their true nature as planes rather than lines.

In a tangential plane cut at an acute angle relative to the Retzius lines (Fig. 7), the Retzius lines appear as broader, rather indistinct zones where the cross-cut prisms may show an altered size and shape (Fig. 8).

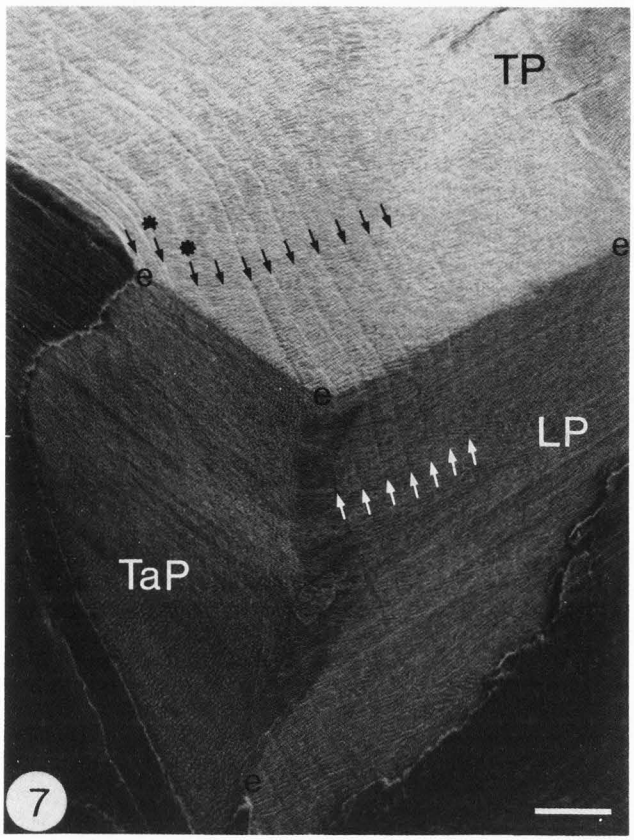
Likewise, in a tangential plane cut at right angles to the prisms and about 45 to the Retzius lines (Fig. 9), zones of differently etched and shaped prisms (Fig. 10) can be identified as representing Retzius lines by referring to the location and pattern of Retzius lines in the adjoining longitudinal plane (Fig. 9). It is noted that the Retzius lines in the tangential plane do not run parallel with the transversely (horizontally) oriented rows of prisms (Fig. 10).

Fig. 7. Buccal enamel of human upper first premolar sectioned along three planes. Regularly spaced Retzius lines (unlabelled arrows) in the outer enamel are continuous across the edge where the transverse and the longitudinal plane meet. The Retzius lines are less distinct in the tangential plane. Bar = 100 µm.

Fig. 8. Higher magnification of area in Fig. 7 where the transverse and the tangential plane meet. The two Retzius lines marked with white arrows correspond to the two Retzius lines marked with asterisks in Fig. 7. Arrowheads demarcate the corresponding "Retzius zones" in the tangential plane. These can only be followed for a short distance. Bar = 10 µm.

Fig. 9. Labial enamel of human upper cuspid sectioned along a longitudinal and a tangential plane. Regularly spaced Retzius lines are marked with arrows in the longitudinal plane. Bar = 50 µm.

Fig. 10. Appearance in the tangential plane of Retzius lines marked with asterisks in Fig. 9. Open arrows point at Retzius lines. Arrowheads correspond to position of arrowheads in Fig. 9. Note difference in shape and etching pattern of prisms within Retzius lines compared to the zone between the Retzius lines. Bar = 10 µm.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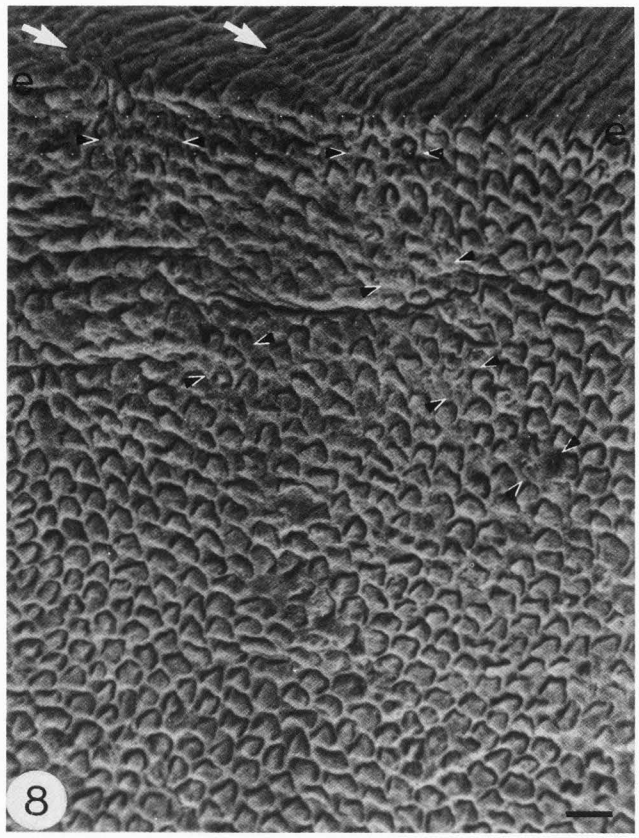
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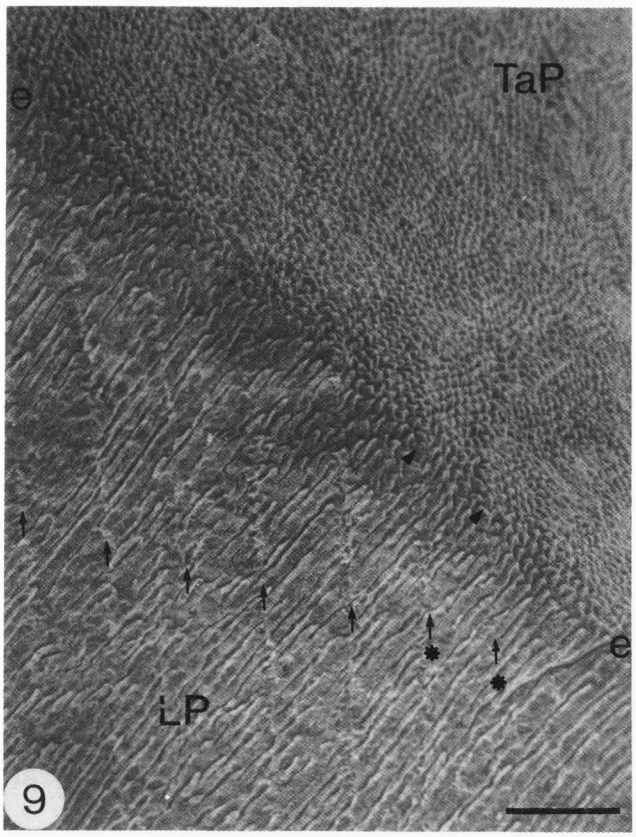
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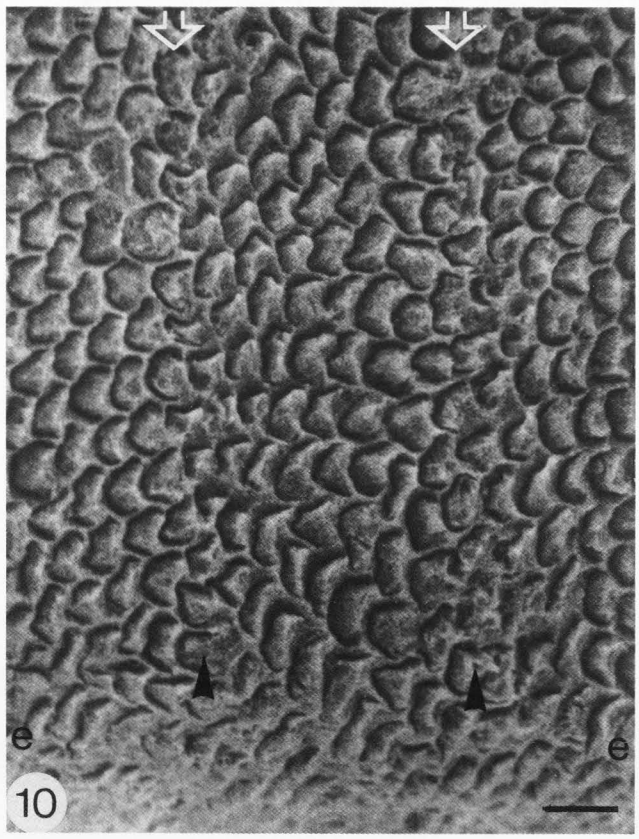
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Prisms. In a plane sectioned perpendicular to the prisms on the labial aspect of an upper central incisor, the shape and etch pattern of prisms at different distances from the dentine-enamel junction is demonstrated (Figs. 11-14). Close to the surface, prisms in large areas are uniformly etched, showing a protruding triangular shape with preferential etching of prism peripheries (Fig. 11). Somewhat deeper and farther incisally, the prisms are also uniformly etched, but here the prism centers tend to be etched more than the interprismatic substance (Fig. 12). By referring to the adjoining longitudinal plane, it is obvious that the direction of prisms relative to the plane of section is almost identical in the two regions. Still deeper and farther incisally, the etching of prism profiles becomes more varied and markedly affected by the presence of Hunter-Schreger bands as observed in the adjoining longitudinal plane (Fig. 13). The same is the case for the region close to the dentine, but variations unrelated to the Hunter-Schreger bands are also seen (Fig. 14).

Tufts. The three-dimensional extent of enamel tufts is well demonstrated where a longitudinal plane meets a tangential plane cut at right angles to the prisms (Fig. 15). The tufts may branch. The transverse waves of the tufts are to a certain extent in phase in relation to the Hunter-Schreger bands. It appears that the tufts encompass both prisms and interprismatic substance (Fig. 16). Longitudinally sectioned prisms situated within tufts are obscured by a veil-like structure, which contains fine striations running in the same direction as the interprismatic crystals in the adjacent "normal" enamel.

Discussion

Our present theories concerning the three-dimensional structure of mature dental enamel are mainly based on light microscopic studies of two-dimensional ground sections (for references see Gustafson (1945) and Boyde (1964)). In addition, methods aiming more directly at three-dimensional structure analysis have been employed; serial grinding (Schnizer, 1925; Freiberg, 1939; Süss, 1940; Fosse, 1968), serial sectioning of partly demineralized enamel (Helmcke, 1964; Warshawsky and Smith, 1971), serial optical sectioning (Osborn, 1967), serial etching (Fosse, 1968), multiplane sectioning-incident light microscopy (Fosse, 1968; Fosse et al., 1973), incident light stereomicroscopy (Sognnaes, 1949; Risnes, 1973), fracturing-scanning electron microscopy (Boyde, 1964, 1969; Skobe and Stern, 1980; Fejerskov and Thylstrup, 1986), photogrammetry of stereo pair images (Boyde, 1966, 1973). The first use of the SEM for simultaneous observation of different planes cut through the same enamel specimen was reported by Fosse et al. (1973). New insight in enamel structure has been gained from studies of thin sections in the transmission electron microscope. However, aside from the difficulties associated with sectioning or rather fracturing ultrathin slices of enamel, TEM studies of enamel thin sections will only image two-dimensional relations, and the field of view will be very restricted.

The scanning electron microscope allows enamel structure to be observed in different planes cut through the same bulk specimen. By tilting the specimen, the various planes can be observed alone or simultaneously. From an overview image structural details may be selected and studied at higher magnification. This unique combination , great depth of focus and high resolution, makes the scanning electron microscope a powerful instrument

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Fig. 11. Labial enamel of human upper central incisor sectioned along two planes. Superficial enamel. Prism peripheries preferentially etched. Bar = 50 µm.

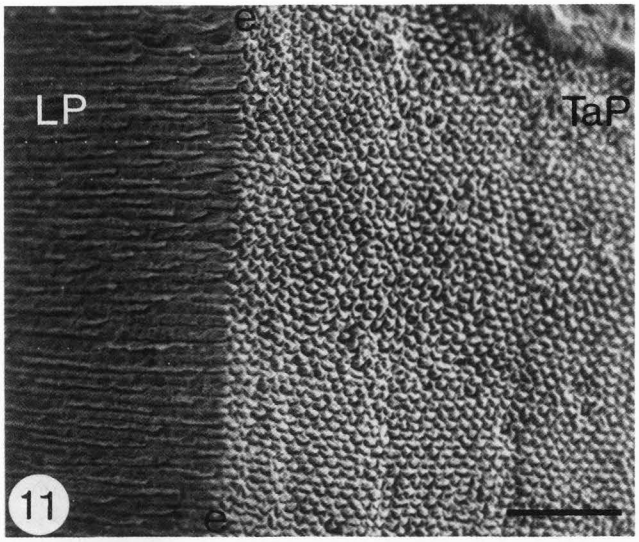
Fig. 12. Same tooth and planes as in Fig. 11, but deeper within the enamel layer and farther incisally. Preferential etching of prism centers. Bar = 50 µm.

Fig. 13. Same tooth and planes as in Fig. 12, but still deeper and farther incisally. Hunter-Schreger bands with alternate zones of transversely and longitudinally sectioned prisms are visible in the longitudinal plane. The variablity in the etching pattern in the tangential plane is clearly related to the Hunter-Schreger bands. Bar = 50 µm.

Fig. 14. Same tooth and planes as in Fig. 13, zone close to dentine. Etching pattern unrelated to the Hunter-Schreger bands is evident (arrows). A is an artifact caused by penetration of glue through crack. Bar = 50 µm.

Fig. 15. Human enamel close to dentine sectioned along a longitudinal plane, and a tangential plane perpendicular to the prisms. One of the enamel tufts can be observed in both planes giving information about its three-dimensional extent. Bar = 50 µm.

Fig. 16. Higher magnification of squared area in Fig. 15. Prisms and interprismatic substance are discernible within tuft, but have a smoothed-out appearance and altered etch pattern. Striation within tuft (white arrows) has same direction as the crystals of the interprismatic substance in the adjacent "normal" enamel (black arrows). Bar = 10 µm.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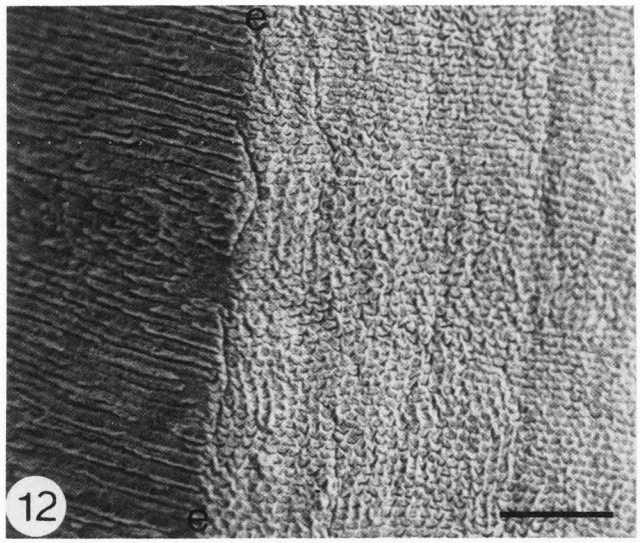
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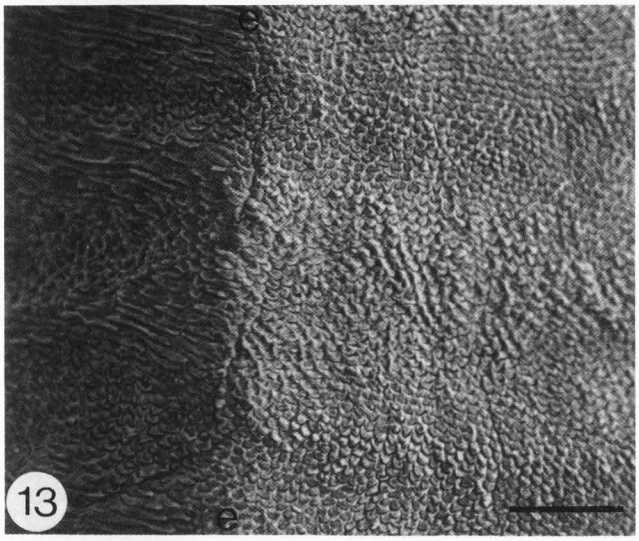
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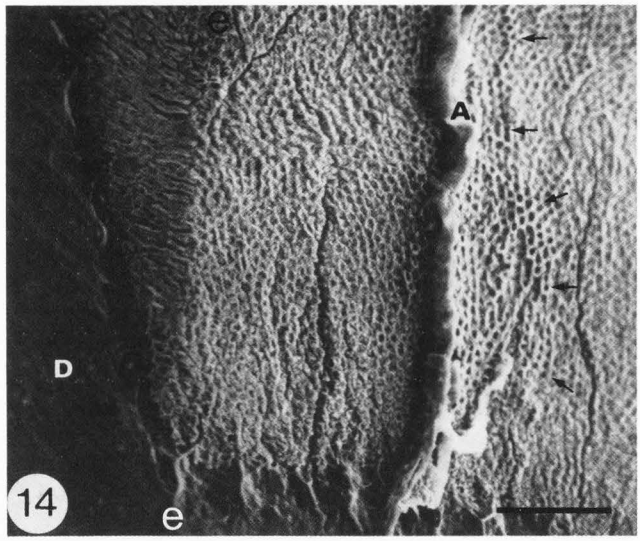
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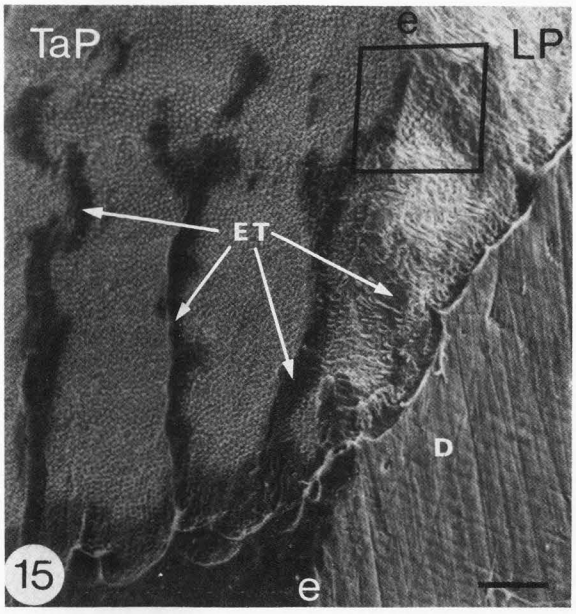
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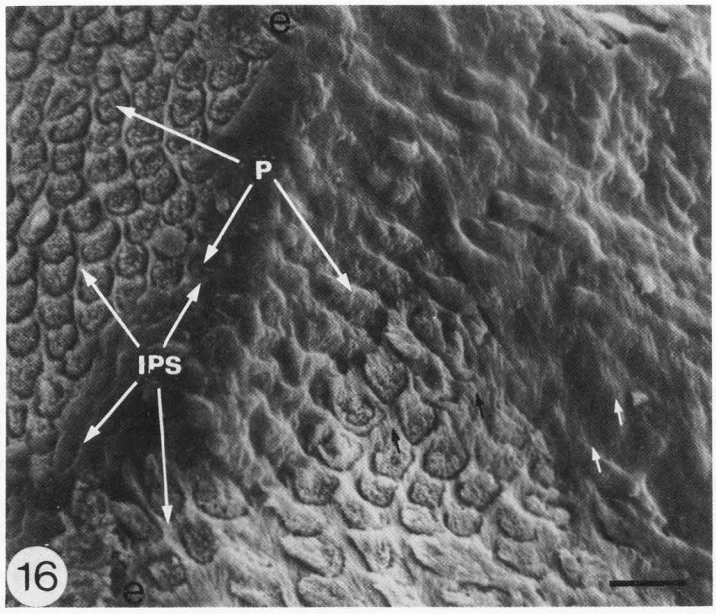
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in the study of three-dimensional aspects of enamel structure. The possibility to directly relate the structure as seen in one plane to the structure in an adjoining plane, makes interpretation of three-dimensional structure easier and safer.

Contributions toward a description of the three-dimensional structure of rat incisor enamel has been offered by a number of authors (e.g. Tomes, 1850; Korvenkontio, 1934; Butcher, 1956; Bouyssou et al., 1962; Helmcke and Rau, 1962; Boyde, 1964, 1969; Weber, 1965; Warshawsky, 1971; Warshawsky and Smith, 1971; Risnes, 1979; Jodaikin et al., 1984). With the MPS-SEM method described here, the three-dimensional structure of rat incisor enamel is more directly accessible, allowing instant check of the relationship between course and appearance of prisms (Figs. 2, 6).

The present method allowed a direct demonstration of the three-dimensional extent of Retzius lines (Risnes, 1985b). The observed continuity of Retzius lines between perpendicular planes excludes the possibility that Retzius lines are border phenomena between rows or layers of decussating prisms (Warshawsky et al., 1984; Warshawsky, 1985). The Retzius lines cross the prisms, which at this site may show an altered size, shape or etching pattern when viewed in cross-section.

The finding that the etching pattern of cross-cut prism profiles varies considerably within the same tangential tooth section, indicates that intraenamel factor(s) other than prism/crystal direction (Simmelink et al., 1974) can modify the etching pattern (Silverstone et al., 1975); this should be born in mind when the effect of different etching procedures are evaluated (Marshall et al., 1975). Since the etching pattern may also affect the appearance of the prism pattern, this parameter should be used with care in characterizing enamel structure for taxonomic or comparative purposes (Boyde and Martin, 1982).

With the present method the enamel tufts can be studied with respect to three-dimensional extent, relationship to Hunter-Schreger bands and relationship to prisms and interprismatic substance.

Conclusions

With the procedures outlined here, involving mechanical preparation (sectioning/grinding), etching, dehydration, coating, and observing in the SEM, a simple, quick and controllable method for analyzing the three-dimensional structure of mature dental enamel is available. With a systematic use of this method, for instance combined with serial etching or serial grinding, a thorough mapping of the three-dimensional structure of dental enamel would be possible.

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Discussion with Reviewers

Reviewer I: The cross-striations are supposed to be diurnal variations in secretion of rods; and striae of Retzius are supposed to be longer interval variations in rod secretion. Thus, striae should only be seen on rods cut in longitudinal section. Can the author explain why he sees them continuous from that plane, to a plane where the rods are cut in cross-section, and the striae now appear to run between groups of rods?

Author: In my opinion the Retzius lines cross the prisms, and where they cross the prisms the structure of the prism/-interprismatic substance is altered. This altered structure is detectable in acid-etched specimens irrespective of the plane of section in which prisms are viewed. In planes where the prisms are cross-cut the Retzius lines are detectable because of an altered structure of the prisms/interprismatic substance (Fig. 10). Thus, the Retzius lines involve the prisms, they are not running between groups of prisms. The suggestion that the Retzius lines represent border phenomena between groups of differently oriented prisms is geometrically impossible. The reason is as follows: Suppose that the lines marked with arrows on the transverse plane (TP) of Fig. 17 (higher magnification of Fig. 7) are border lines between different groups of prisms, G1, G2, G3, and G4. Favourably judged these groups encompass not more than 1-3 prisms. Consequently, border lines separating correspondingly small groups

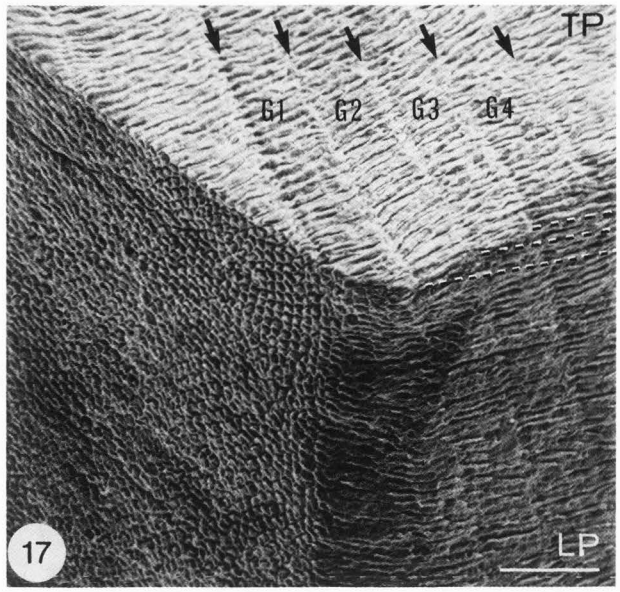

Fig. 17. Higher magnification of Fig. 7. If the Retzius lines (arrows) in the transverse plane (TP) are border lines separating different groups of prisms (G1-G4), these groups would have to be delimited by lines running like the stippled lines in the longitudinal plane (LP). Bar = 50 µm.

of prisms should be running along the stippled lines in the longitudinal plane (LP) (Fig. 17). No such lines could be observed, however. The same reasoning can be applied to Fig. 9.

Reviewer IV: In your paper you refer to the studies by Warshawsky et al. (1984) where they suggest that the three-dimensional organization of crystals imposes the repetition necessary to produce rhythmic patterns. You are studying surfaces which have been sectioned and etched. How can you be sure that the Retzius lines you are showing are not a reflection of the crystal arrangement?

Author: I believe that the Retzius lines are due to an altered structure of prisms/interprismatic substance, and in that sense they reflect the crystal arrangement.

Reviewer IV: In your fig. 7 at the surface marked TP it is possible to discern lines running in parallel but crossing your Retzius lines in an angle of about 45° . This phenomenon is in particular evident in the area below the arrows. How do you explain this phenomenon?

Author: The lines visible below the black arrows are probably remnants of scratches created during the sectioning/grinding procedures. There is also one in the upper left corner of Fig. 7, but this has a different direction.