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HIGH RESOLUTION ELECTRON MICROSCOPY OF ENAMEL CRYSTALLITES DEMINERALIZED BY INITIAL DENTAL CARIES

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Abstract

Acids produced by various oral bacteria cause mineral loss and crystallite dissolution during the development of enamel caries. In order to demonstrate this phenomenon, the initial disappearance of lattice fringes and the formation of a central perforation in crystallites were examined by high resolution electron microscopy (HREM) in initial enamel caries without macroscopic tissue evidence of destruction. Ultrathin sections were also examined by selected area electron diffraction to reveal the mineral phase of the surface layer in carious enamel.

A marked variation in the dissolution pattern was disclosed in the initial carious lesions. HREM revealed that disappearance of the lattice fringes from the lateral portion of the crystallites was predominant in the superficial layer covering the lesion, while central perforation of crystallites mainly occurred in the subsurface prismatic region. The beginning of the central dissolution occurred at the dislocated area where lattice striations appeared to be disordered. Selected area electron diffraction of the gradually demineralized enamel revealed a pattern consistent with hydroxyapatite (OH-AP) or fluorapatite (F-AP) mineral.

These findings suggest that the susceptibility to caries of enamel crystallites is spatially and temporally different during the progression of the caries. Furthermore, the formation of central perforations and the consequent easy intracrystalline diffusion of acids might induce rapid crystallite dissolution.

Key Words: Dental caries, enamel crystallite, initial dissolution, high resolution electron microscopy, selected area electron diffraction, hydroxyapatite, fluorapatite, lattice fringes, interface, dislocation.

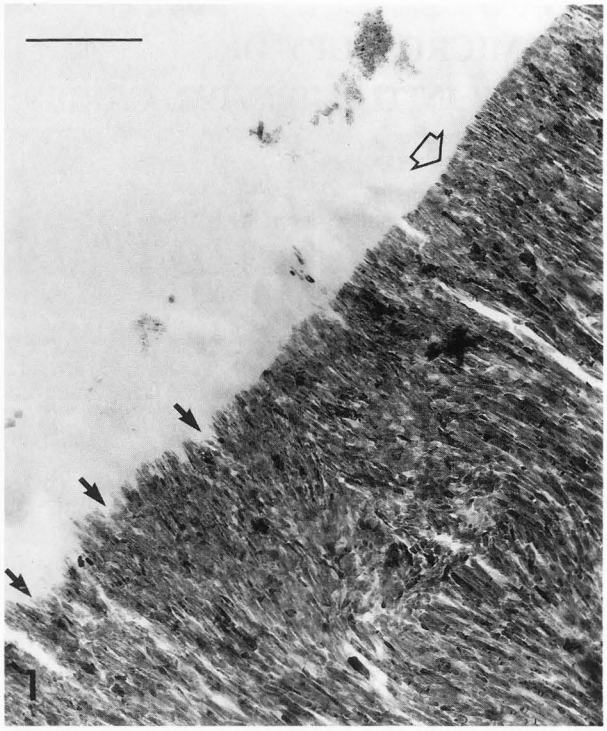
Introduction

Using transmission electron microscopy (TEM), Frank and Brendal [3] reported that two types of defects were located in the surface layer covering a carious lesion. The first defect was superficial while the second consisted of a very small and deep defect which extended through the almost intact external surface layer to reach the subsurface enamel lesion. The first HREM of enamel caries revealed two types of crystallite dissolution in the white and brown spots of carious enamel [19]. The first consisted of a central dissolution which developed strictly along the (100) and (010) planes parallel to the c-axis; the second started at the external lateral surface with prevailing development along the a- and b-axes of the crystallite.

Recently, lattice fringes and atomic structures in synthetic apatites and enamel crystallites have been observed in the modern high resolution TEM (HRTEM) with a point-to-point resolution of better than 0.25 nm [13, 15, 16, 17]. Crystallite association at the lattice striation level has also been demonstrated using a similar technique [6, 7, 8]. The present study was designed to spatially investigate the dissolutive processes in the initial enamel caries using a HRTEM operating at an accelerating voltage of 300 kV.

Materials and Methods

A maxillary third molar of a 23-year-old man was used. Its occlusal plane with carious destruction was exposed to the oral cavity. After extraction, 9 pieces of occlusal enamel having a varying degree of brown spot-like lesions about 2 mm in width were excised with a diamond disk under a stream of Ringer's solution. They were immersed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 24 hours, postfixed in 2% osmium tetroxide in the same buffer for 1 hour and then dehydrated in increasing concentrations of ethanol and embedded in epoxy resin. Ultrathin sections were cut with a MT-5000 ultramicrotome equipped with a diamond knife and collected in a specimen boat



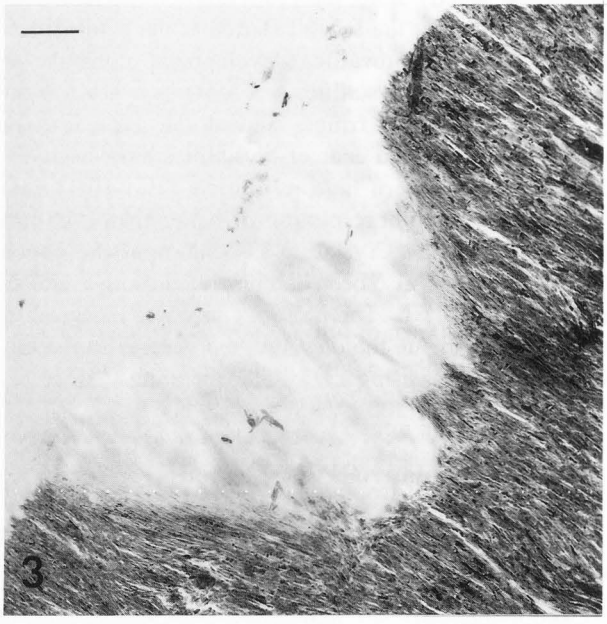


Figure 1 (top). Initial carious lesion of enamel surface. The margin (arrows) is continuous with the intact area (open arrow). Bar = 1 μm.

Figure 3 (bottom). Enamel loss at electron microscopic level. Bacteria occupy the defect which has irregular margin. Bar = 1 μm.

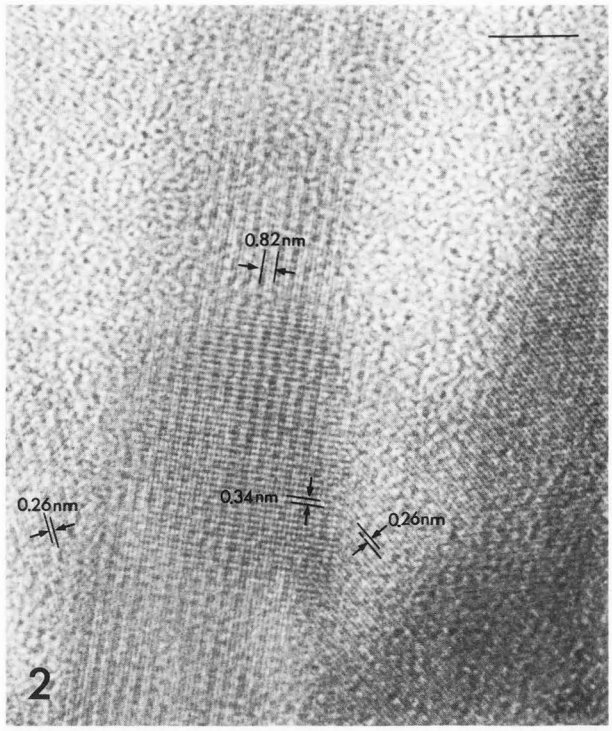


Figure 2 (above). High resolution image of superficial intact crystallites. Lattice striations in the central crystallite, with 0.34 nm intervals, are in contact with those in the right crystallite. The fine crystallite with lattice fringes with 0.26 nm intervals also is in contact with the central crystallite. Bar = 5 nm.

Figures 4 and 5 at facing page 201 column 1.

Figure 4. High resolution image of partially dissolved crystallite at the surface of an enamel indentation. Note the irregular margin of the crystallite. Bar = 5 nm.

Figure 5. Carious enamel without any defect. Note the most external layer measuring about 1 μm in width without clear enamel dissolution. Bar = 1 μm.

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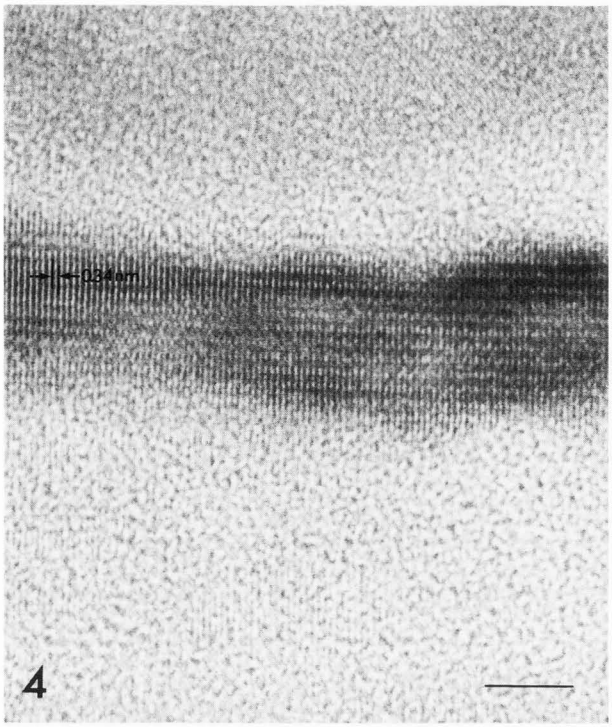
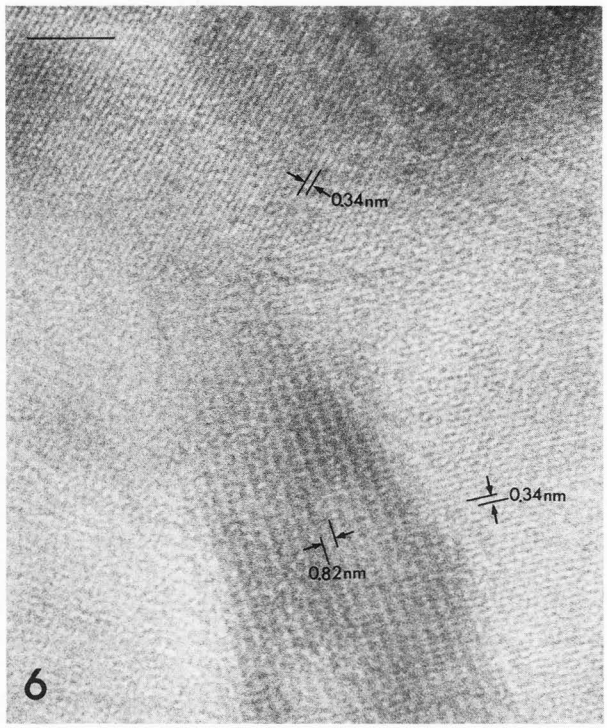
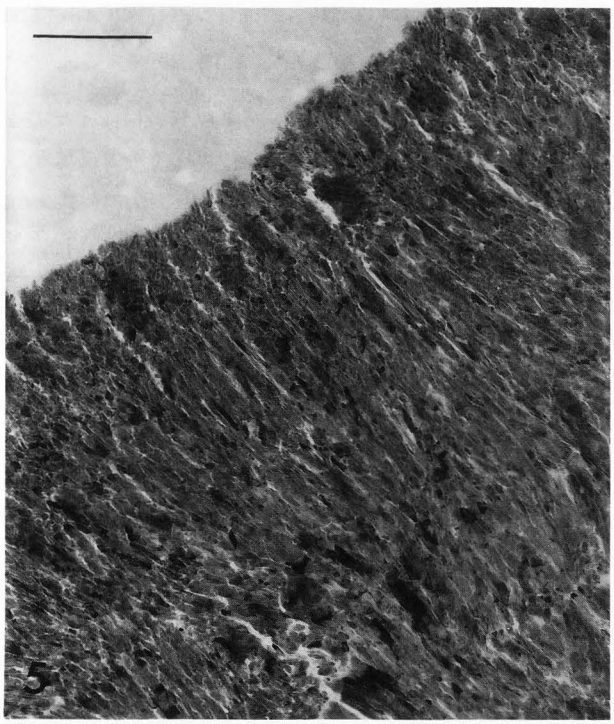
containing a saturated solution of human dentine powder to prevent section demineralization [5]. They were mounted on Formvar-coated 300-mesh copper grids reinforced with carbon about 5 nm in thickness and examined without electron staining in a Hitachi H-9000 electron microscope operated at an accelerated voltage of 300 kV [7].

Furthermore, ultrathin sections were used for selected area electron diffraction to identify the mineral phase of the carious enamel. The d-spacings of the diffraction patterns were calibrated from those of gold obtained under identical conditions.







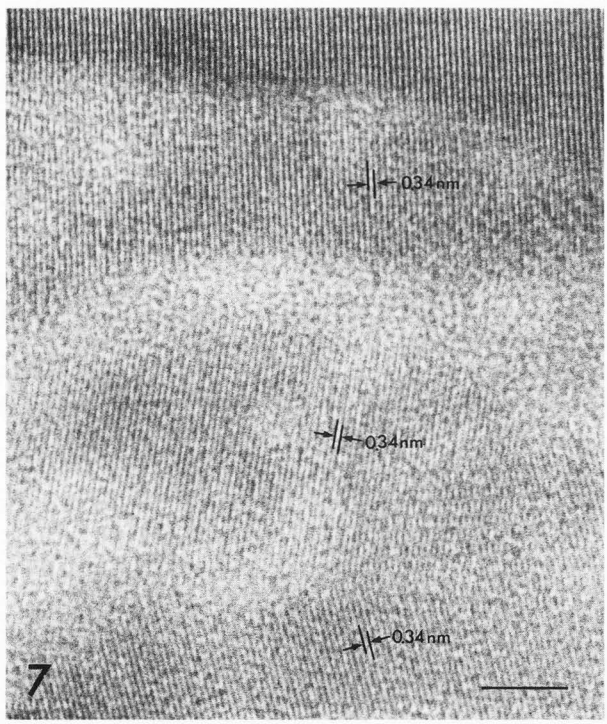


Figure 6. High resolution image of the external layer. The lattice fringes of a partially demineralized crystallite, with 0.82 nm intervals, are in contact with those of adjacent crystallites. Bar = 5 nm.

Figure 7. High resolution image of fine crystallites at the superficial layer. Note two fine crystallites (central and lower parts of the figure) adjacent to the enamel crystallite (upper part of the figure). Bar = 5 nm.

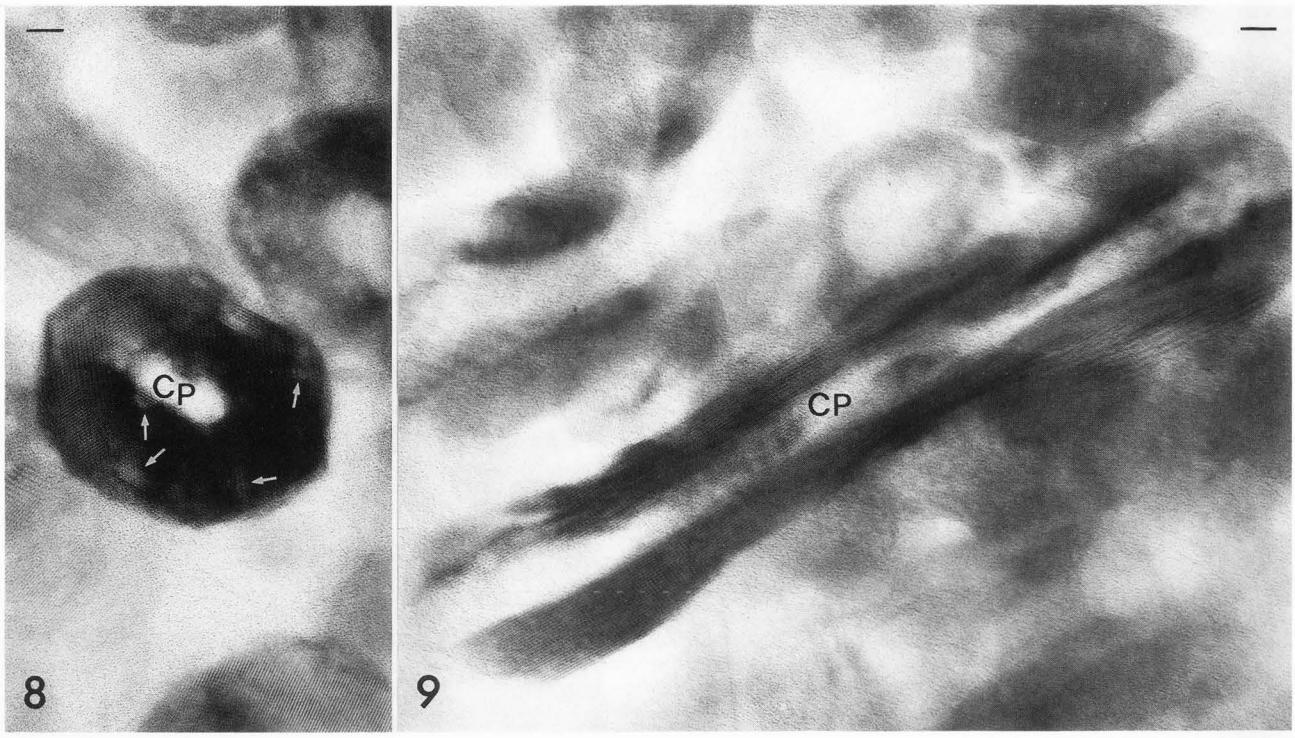


Figure 8. High magnification image of the central perforation (CP) of a demineralized crystallite (cross-section). The arrows indicate small electron-lucent areas. Bar = 10 nm.

Figure 9. High magnification image of the central, tunnel-like perforation (CP) of a demineralized crystallite (vertical section). Bar = 10 nm.

Figures 10a and 10b (on the facing page). High resolution electron micrographs of initial central perforation. **(a)** High magnification image of a demineralized crystallite. Note the central electron-lucent area (arrow). Bar = 10 nm. **(b)** High resolution image of the electron lucent area (arrow in a). The dislocated area (bracket area) shows an irregular configuration of the lattice fringes. This figure shows the typical (001) plane of a single crystallite. Bar = 5 nm.

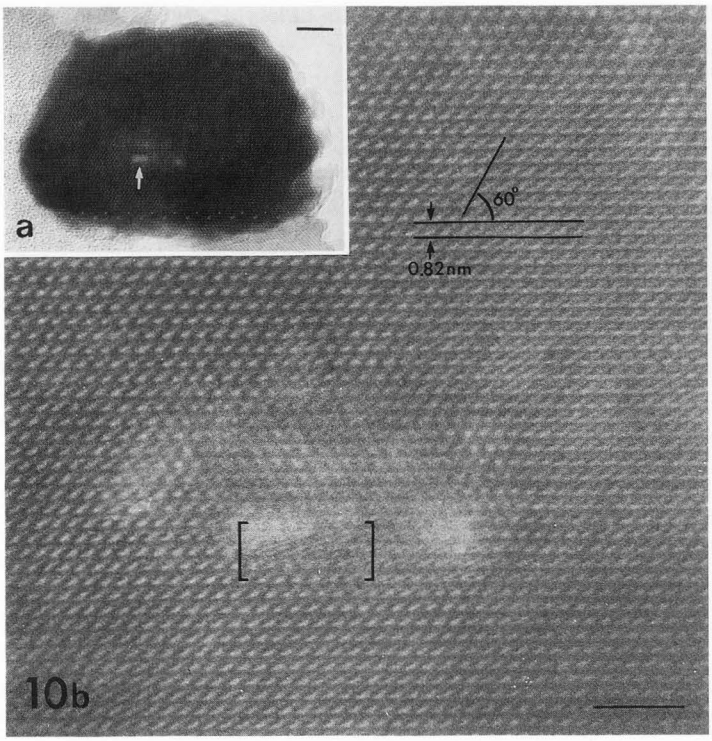
Figure 11 (on the facing page). High magnification image of demineralized crystallites; the open arrows indicate the associated region between dissolved crystallites. The damaged lattice image is seen along the central part of a demineralized crystallite (large closed arrow). The small arrow shows the tearing region of the lattice fringe. Bar = 10 nm.

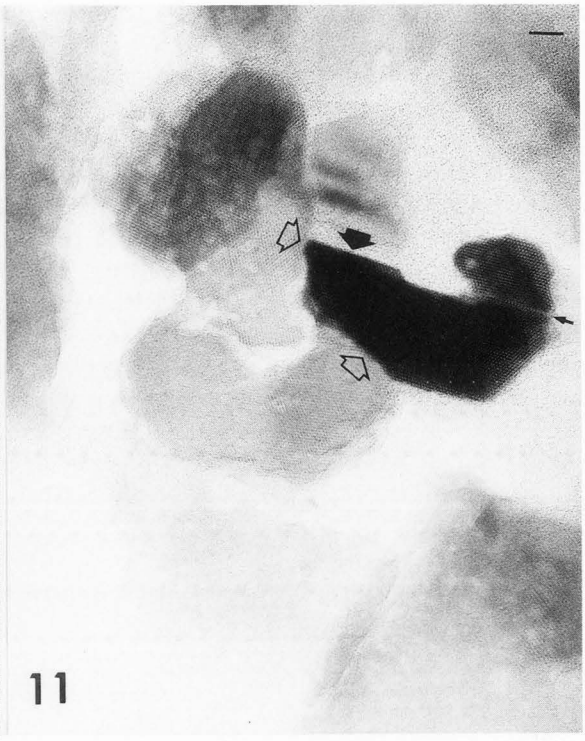
Results

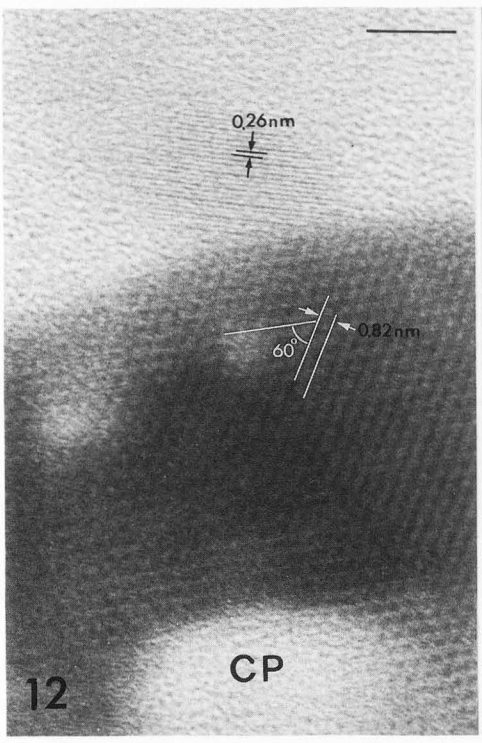
The intact enamel surface investigated was smooth and covered by an extremely thin layer of dental plaque when observed at low magnification (Fig. 1). The enamel crystallites positioned closely side by side had an intimate association with each other. Furthermore, fine crystallites associated at the side of enamel crystallites (Fig. 2). A tilt boundary between lattice striations was identified at such junctions. Although, at low magnification, the initial changes obviously caused by a carious attack were observed at the fluffy margin of superficial enamel crystallites (Fig. 1), HREM revealed that the crystallites positioned side by side still had an intimate association in their lower portion.

The carious changes at the enamel surface showed two different patterns. In one case, enamel dissolution was represented by microscopic indentations, the largest being about 5 μm in depth (Fig. 3). Although bacteria were seen within these cup-like defects, crystallite demineralization was restricted at their periphery (about 0.8 μm in width) where thin enamel crystallites were predominant. HREM indicated that the thin appearance of the crystallites was due to a partial disappearance of their lattice fringes along their lateral surface (Fig. 4).

The second pattern of carious change was characterized by the dissolution of the enamel crystallites under a thick layer of dental plaque without clear electron-microscopic demonstration of enamel loss. A typical image of this type of dissolution is shown in Figure 5. In







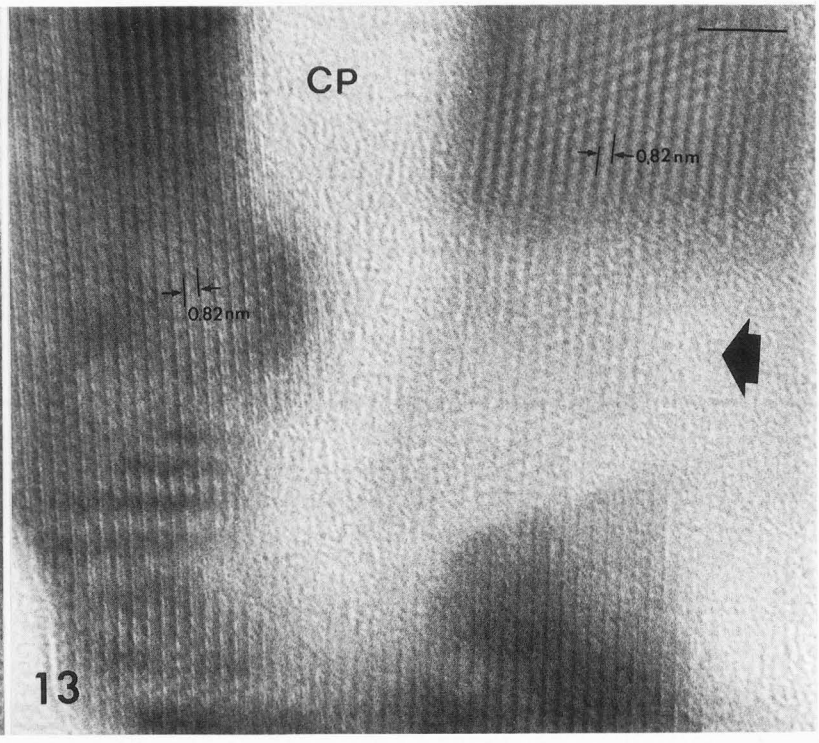


Figure 12. High resolution image of the junction between the dissolved crystallite [(001) plane] having a central perforation (CP) and a fine crystallite having lattice striations with 0.26 nm intervals. Bar = 5 nm.

Figure 13. High resolution image of the region between the inside (CP) and outside (arrow) of a dissolved crystallite. Bar = 5 nm.

this case, a lesion extending to about 300 μm in depth was observed under a dissecting microscope. An external layer about 1 μm thick was demineralized. The HREM showed partial disappearance of the lattice fringes at the tip and lateral surface of demineralized crystallites (Fig. 6). A curvature of lattice fringes was recognized at the interface. The enamel crystallites in the superficial layer (except the external, 1 μm thick part) were positioned side by side with their long axes mostly perpendicular to the surface. HREM revealed that although a partial disappearance of the lattice fringes was observed at the lateral surface of some crystallites, the association between the lattice striations in the intact enamel crystallites was still recognizable. Furthermore, fine crystallites were also recognized adjacent to the enamel crystallites (Fig. 7). In addition, intricate and irregular boundaries were located at the interface between these deposits.

Prismatic structures were visible in the enamel located about 25 μm below the carious surface. The most characteristic trait in this region was a central perforation of a number of crystallites having lateral dissolution (Figs. 8 and 9). These changes were investigated mainly according to the (001) plane of a single crystallite. HREM revealed that the initial central dissolution occurred at the dislocated areas where the lattice striations appeared to be disordered (Fig. 10). Although an association between the lattice fringes of demineralized crystallites was frequently observed (Fig. 11), a coincidence between a fine crystallite and a demineralized crystallite was very rare in this region (Fig. 12). Besides the central perforation, a lateral perforation was sometimes observed. It produced a connection between the central and the lateral parts of the enamel crystallites (Fig. 13). Furthermore, a damaged lattice image, such as that of demineralized crystallites, was seen along the central part of the crystallites (Fig. 11). The selected area electron diffraction revealed the well-known pattern of concentric rings. Five rings obtained from the external, about 1 μm thick, layer had d-spacings of 0.344, 0.316, 0.281, 0.264 and 0.184 nm. Three rings obtained from the superficial layer (except for the external part) had d-spacings of 0.344, 0.281 and 0.173 nm. Three rings obtained from the subsurface prismatic region had d-spacings of 0.308, 0.281 and 0.264 nm. These d-spacings were consistent with OH-AP or F-AP.

Discussion

Electron microscopy shows that sound and demineralized regions coexist at the limited area where enamel surface is macroscopically smooth and covered by a thin layer of plaque. This finding thus indicates that no special structured spots indicative of caries initiation exist. The initial dissolution is not uniform because the bacterial colonization is not uniform and/or there may be some actual chemical difference in the composition of the various crystallites. The roughness of the enamel surface caused by a carious attack might therefore be convenient for the mechanical retention of the plaque. As plaque retention subsequently accelerates crystallite dissolution, this phenomenon might cause a superficial defect on the outer enamel layer. In this type of superficial dissolution, demineralization occurs predominantly at the lateral crystallite surface.

After the caries is formed, enamel dissolution can occur at three different layers: the superficial demineralized layer, the subsurface demineralized layer, and the perforated crystallite layer at the prismatic level. The main difference from the enamel defects mentioned above was a thick layer of plaque and the gradual demineralization under the plaque. In the surface layer of enamel, partial disappearance of lattice striations at the tip and lateral surface of the crystallites was observed, but the extensive crystallite dissolution found in the subsurface prismatic layer was lacking. This finding seems to indicate that the surface layer has some caries resistance. This might be due to the presence of F-AP. The present selected area electron diffraction patterns obtained from the superficial carious enamel could not distinguish OH-AP from F-AP; however, an X-ray microanalysis study demonstrated that high concentration of fluoride (F) was present at the superficial layer (10-25 μm thick) of the carious enamel [18]. The incorporation of F in OH-AP promotes the substitution of F for OH. The greater stability of the F-structure is brought about by the hydrogen bond between OH and F [14].

In the present HREM study of crystallite dissolution, the most interesting finding was the central perforation of the subsurface enamel crystallites. This was easily seen in crystallite vertical sections, nearly parallel to the c-axis; it was confirmed in cross-sections of nearly cylindrically demineralized crystallites. Not all demineralized crystallites showed central perforation under the HREM. This means that the physico-chemical resistance against carious attack is different among crystallites even at the same enamel depth. Early stages of central dissolution were observed in single crystallites which showed advanced lateral dissolution. This finding suggests that the lateral dissolution precedes the central dissolution. The central part of the crystallite represents its early formed part [2] where dislocations are a crystallographic characteristic [10, 12]. The existence of central dislocations is thought to be the main reason for the formation of the central perforation [1]. Furthermore, compared with the superficial layer, the F concentration of the subsurface prismatic layer was reported to be low [4, 18, 20]. This would facilitate the progression of the

central dissolution along the c-axis of the crystallite. The demineralized crystallite with central perforation had small electron-lucent areas which were thought to correspond to regions with low resistance to the electron beam [9]. This suggests that the lateral perforation between the inside and outside areas observed after the development of the central perforation might begin at these physically weak regions of crystallites. Subsequently, carious enamel crystallites would be completely dissolved; the central perforation might favor the diffusion of acids within the crystallites, thus promoting their rapid dissolution.

The lateral association between perforated crystallites and fine crystallites was very rarely observed in the present study. The size of the latter crystallites was very small and no demineralized hollow was observed at the periphery of the former. At a high resolution electron microscopic level, these findings indicate that the fine crystallite might be formed *ex novo* by some remineralization process. The surface layer of the enamel lesions has been reported to be a presumed obstacle for remineralization [11]. The superficial layer found in the present study might therefore inhibit salivary remineralization of the subsurface lesions.

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Discussion with Reviewers

D.G.A. Nelson: This investigation was based on one tooth from one person. Have other teeth and other lesions been investigated?

Author: As the goal is to prevent dental caries and/or to promote the natural healing of initial carious lesions, my study is now focused on the initial lesions. The lesions including macroscopic defects of enamel surface were intentionally excluded in the present investigation. I think the present material is sufficient for the study of initial enamel caries because various brown spots of the enamel surface were thoroughly examined.

D.G.A. Nelson: Are the "fine crystallites" observed in the surface layer the results of the remineralized process?

Author: The fine crystallites are probably newly formed and are similar to those observed in the initial dental calculus formation originating from saliva. This situation can be considered a sort of remineralization process.

D.G.A. Nelson: What was done to differentiate the effect of electron beam damage from "real" acid dissolution?
Author: If one crystallite is overexposed to the electron beam, its electron density rapidly decreases at apical regions to form electron-lucent areas. In this case, the lattice fringes are still recognized at the beam-damaged areas. On the contrary, lattice fringes cannot be observed in the lesions due to "real" acid dissolution, as shown by the appearance in their background of the nearly atomic image of epoxy resin.

E. Bonucci: What does the author think about the cause of axial dislocations and, especially, about the hypothesis that an axial organic component favors the longitudinal crystal perforation?
Author: The beginning of the crystallite growth occurs at the central part of the crystallite. This process starts through the interaction between organic and inorganic substances. The appearance of inorganic phases is thought to cause complicated changes at the atomic level: structural defects and substitution of some ions. These phenomena are probably at the origin of axial crystallographic dislocations and central perforations.

Reviewer IV: What the author calls "fine crystallites" may well be regions that, because of the topography of the plane, are more or less out of focus, which yields some lattice fringes or no fringes at all. The author should consider this possibility before postulating events that may not have happened during caries formation.
Author: In my opinion the figures were taken at proper focus, because in the present study the electron microscope was used at 300 kV and the thickness of the sections was around 70-80 nm. The running direction of newly formed crystallites is different from that of enamel crystallites. Even though the focus problem exists, the total image of these tiny crystallites could be identified on the negative films.

Reviewer IV: The separation into two types of caries is arbitrary; is the author aware that he could be describing two different manifestations of the same process?
Author: Dental caries is clinically classified into two groups: acute and chronic patterns. Although the dissolution process by bacteria-produced acids is same, the spreading process of caries is thought to be different mainly due to the degree of plaque retention and tooth susceptibility to acids. The present HREM study would directly demonstrate two different manifestations of the enamel dissolution at the superficial layer in the initial carious lesions.