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The Dentino-Enamel Junction: A Structural and Microanalytical Study of Early Mineralization

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Summary. The spatial localization of enamel and dentin apatite crystals of the rat tooth has been studied by electron microscopic methods—bright field, selected-area dark field,and electron spectroscopic imaging. The sequential events of dentin calcification followed by the formation and growth of enamel crystals were determined and compared to previous studies. In dentin, initial sites of mineral deposition occur in areas subjacent to the dentino-enamel junction (DEJ). The subsequent expansion of these deposits progresses towards the DEJ to the terminal ends of dentin collagen fibrils. Concomitantly, an electron-dense enamel matrix is released by ameloblasts; with the presence of this matrix, the growth of enamel crystals occurs from the underlying calcified dentin. Enamel crystal growth continues to within close proximity of the plasma membrane of ameloblasts. A close spatial relationship between enamel and the crystals of calcified dentin collagen fibrils was observed by selected-area dark field imaging. Such areas of crystal intimacy show a co-localization of calcium and phosphorus extending from calcified collagen fibrils to enamel sheaths which encase enamel crystals. A working model of the spatial relationship between crystals of dentin and enamel is presented and discussed in light of mechanisms by which calcified dentin may promote the formation of enamel crystals.

Key words: Tooth enamel — Dentin — Apatite crystals — Ultrastructure — Selected-area dark field imaging — Electron spectroscopic imaging.

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Amelogenesis, the formation of tooth enamel, can be classified into several different stages: induction, presecretory, secretory, and maturation. Transition between each of these stages is thought to be a result of inductive interactions between both cellular and extracellular components of enamel and dentin [1]. This developmental process has been the subject of studies that have included matrix chemistry [2–4], microscopic analyses [5–11], ion transport mechanisms [12–14], and cell dynamics [15].

The basis for our interpretation of the initiation of enamel crystals was made by electron microscopic studies which illustrated the structural events of this process as it occurs early in the secretory stage [16–18]. A close spatial and temporal interrelationship between the crystals of enamel and dentin is suggested by the apposition of crystals at the DEJ and the advancement of enamel crystal growth from the calcified dentin towards the overlying ameloblasts.

Although both enamel and dentin matrices have an intimate organic/inorganic interface, their structure, chemistry, and crystal order are very different. Factors within the fibrillar and extrafibrillar dentin matrix limit the apatite to a small crystal size but allow for their very random ordering. In enamel, the opposite is true as very large crystals are highly organized within rod and interrod domains. This varied crystal ordering is reflected by the different chemistry of these two matrices [2, 19].

The present study was undertaken to reexamine the structural and chemical features of initial enamel crystal formation at the DEJ on aqueously and non-aqueously fixed tissues using, in addition to bright field electron microscopy, selected-area dark field imaging and electron spectroscopic imaging. The former technique was used to provide the specific and direct visualization of apatite crystals [20–22] and the latter was used to obtain high reso-

lution elemental maps of phosphorus and calcium [23, 24]. The results of this study show the sequential events of dentin calcification and enamel crystal formation, the specific localization of early enamel apatite crystals, and the spatial co-localization of phosphorus and calcium in the immediate environment of the developing enamel crystals contained within enamel sheaths. The implications of these results are discussed in light of a working model of the spatial relationships between dentin and developing enamel crystals.

Materials and Methods

Incisors and molars of 3 to 7-day-old Sprague-Dawley rats were removed under general anesthetic conditions. The incisors were allowed to air-dry for several weeks and were then directly embedded in Spurr resin under low vacuum without the use of routine microscopic fixation and solvents. Molars were removed and sliced in half; the cut edges of the enamel and dentin were then slam frozen according to the procedure described by Arsenault et al. [24]. Freeze substitution was carried out in methanol containing 0.5% glutaraldehyde at −85°C for 48 hours followed by a gradual increase to room temperature. Specimens were embedded in Spurr resin. Also, rats were perfused with buffered glutaraldehyde; molars were excised and osmium post-fixed, dehydrated, and resin embedded. Thin sections were cut with a diamond knife and floated onto either distilled water or a methanol-glycerol mixture (1:1; v:v). Sections for examining matrix structure were demineralized in 4% EDTA for 30 minutes and stained in 1% aqueous phosphotungstic acid and 2% aqueous uranyl acetate for 20 minutes each. For bright field and selected-area dark field electron microscopy, individual undemineralized sections were placed on Formvar-coated grids, immediately blotted dry, and examined without staining in a Philips 300 electron microscope operating at 80 kV. Tilt-beam dark field imaging was used to collect selected diffracted electrons from the enamel and dentin apatite crystals; this selected area corresponds to the combined reflections of [(102),(210),(211),(112),(300),(202), and (301) d spacings] and the c-axis [(002) d spacing] of apatite. These axial reflections were imaged collectively with a 10 μm objective aperture with a camera length of 560 mm.

Ultrathin sections for electron spectroscopic imaging were placed on holely plastic-coated grids and examined with a Zeiss 902 electron microscope; this is described in more detail by Arsenault and Ottensmeyer [23]. Elemental maps for P and Ca were obtained by computer subtraction of image sets taken at energy losses of 120/150 and 320/360eV, respectively.

Results

In longitudinal planes of section through the tooth, the sequential events of both dentin and enamel development can be readily observed. Figure 1 is a galley of stained bright field electron micrographs showing sequential stages of transition along this developmental gradient at the DEJ. As a result of routine staining procedures, all detectable calcium/phosphorus and mineral apatite have been depleted [20]; however, the former sites of mineral deposition are discernable by structural modifications of the matrix which accompanies mineralization. Figure 1a shows localized stained densities representing former nascent sites of mineral deposition within the dentin matrix. Such nascent sites are typically found subjacent to the DEJ in association with collagen fibrils; dentin collagen fibrils at the DEJ appear to radiate towards the ameloblast layer. With further development, larger tracts of mineralized dentin form and in stained sections such areas are characterized by an amorphous matrix in which collagen fibrils can be observed (Fig. 1b). The fibrils within these areas are three to four times the width of nonmineralized fibrils. As dentin mineral advances apically, it also expands laterally to the DEJ giving an irregular surface to the dentin caused by projecting terminal ends of mineralized collagen fibrils (Figs. 1b and c). In stained sections, this dentin surface is delineated by intense staining (Fig. 1b–f) which appears analogous to the lamina limitans found in stained mineral deposits of bone and cartilage [25]. Concomitant with dentin mineralization, ameloblasts begin to release an electron-dense matrix (Fig. 1b, c) and by continued secretion their cell apices gradually move away from the dentin surface. With full calcification of the dentin at the DEJ and in the presence of enamel matrix, enamel crystals form at the dentin surface extending towards the overlying ameloblasts (Fig. 1d). These ribbon-like enamel crystals continue their growth to be in close proximity to the ameloblast apical membrane (Fig. 1e). This developmental gradient is schematically shown in Figure 7a.

EDTA-treated/stained sections of the DEJ were used to remove all detectable mineral and to show the underlying structure of the organic matrix in both cryogenic (Fig. 2) and aqueous (Fig. 3) preparations. The former presence of early enamel crystals is evident by a 50 Å lucent space surrounded by a heavily stained thin matrix — the enamel sheath. Similar observations between the enamel crystal and the surrounding organic matrix have been previously made [26, 27]. After prolonged EDTA/staining exposure, the electron-dense interface at the DEJ shown in Figures 1 and 2 was removed illustrating the terminal ends of dentin collagen fibrils (Fig. 3). These terminal ends appear broadened and possess a reduced axial banding pattern; in instances where the banding pattern is absent, these terminal ends appear frayed and somewhat disorganized. Electron-lucent spaces representing the former presence of enamel crystals are found to be directly associated with these expanded colla-

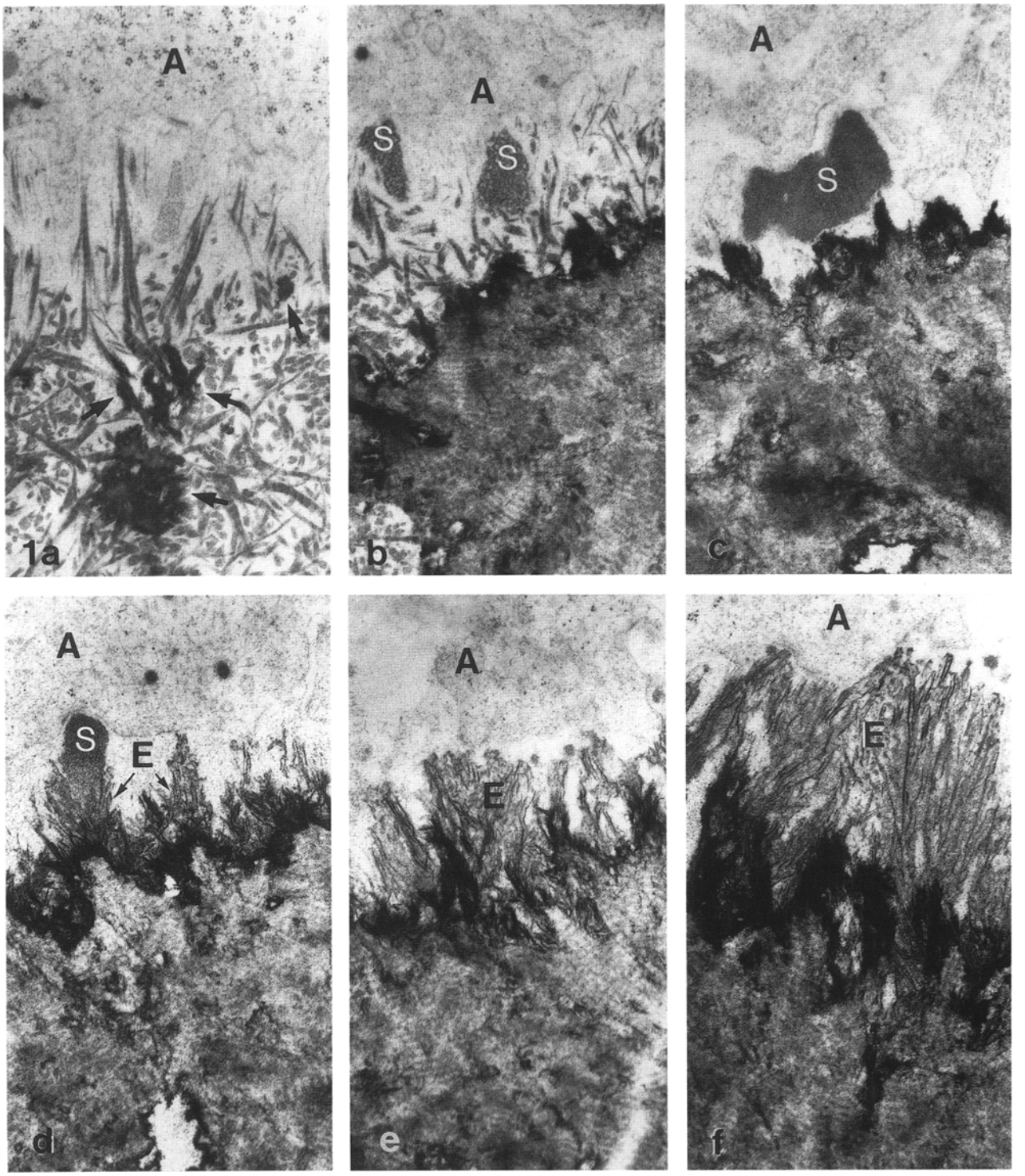

Fig. 1. A galley of stained bright field images showing the sequential steps of dentin calcification, the deposition of enamel matrix, and the formation enamel crystals. **(a)** An early stage of dentin calcification showing centers of mineral deposition (arrows) positioned about collagen fibrils; many fibrils project towards the ameloblast layer (A). **(b)** The demineralized dentin shows large, formerly calcified collagen fibrils; this region is separated from the ameloblasts (A) by a narrow band of non-calcified dentin collagen fibrils. Large, secretory deposits of enamel matrix (S) are found adjacent to the ameloblast cell apices. **(c)** Dentin calcification progresses to the terminal ends of collagen fibrils. **(d)** From the peripheral border of dentin, enamel crystals (E) grow into the secreted enamel matrix (S). **(e,f)** With further development, these enamel crystals (E) grow and become closely associated with the ameloblast (A) cell membrane. Molar, glutaraldehyde perfused. ×28,000.

Fig. 2. An EDTA/stained section of the DEJ shows enamel sheaths (arrowheads) with lucent centers. A dense stained region marks the interface of the DEJ. Molar, slam frozen/freeze substituted. ×70,000.

gen ends both at their terminal and lateral aspects (Fig. 3).

Figure 4a is an unstained bright field image taken at a comparable stage of amelogenesis as that shown for stained sections in Figure 1e and f, and Figure 3. In this image the mineral apatites of both dentin and enamel are visualized by their high native mass density; the organic components of these matrices are not visualized. A close spatial relationship between the crystals of enamel and dentin is apparent at the DEJ. This same area imaged with selected-area dark field (Fig. 4b) reveals a portion of the total apatite crystal distribution within both enamel and dentin. The highlights of this combined a,b + c-axial image represent several different lattice planes of apatite. This combined image was shown to maximize the number of possible reflections due to the random ordering of crystals in dentin and to a certain extent that of enamel. At the DEJ, dentin crystals are easily recognized and are frequently observed in association with enamel crystals projecting into the enamel matrix. These early enamel crystals are 50 Å in width and have a variable c-axial length depending ontheir precise stage of development.

Electron spectroscopic imaging was used to detect the presence and distribution of Ca/P at the DEJ (Fig. 5). Figure 5a, a spectroscopic image taken at 150 eV, is formed by electrons scattered by the native mass densities of both organic and inorganic components of the DEJ. Elemental maps of P and Ca from this same area are shown in Figure 5b and c. As expected, there is a Ca/P co-localization over the calcified areas of dentin collagen fibrils and enamel crystals. However, this co-distribution of Ca/P in the enamel is not restricted to a central portion defined by the apatite crystal but also extends over the entire enamel sheath. The Ca distribution appeared somewhat dispersed within the enamel matrix whereas the P elemental map defined the DEJ morphology and the close spatial relationship between enamel and dentin.

At a later stage of development (Fig. 6), enamel crystals ranging up to 600 Å wide can be easily distinguished from the smaller dentin crystals measuring 110–150 Å long by 50 Å wide. These enamel apatite crystals terminate and interdigitate with the underlying dentin layer. Apatite lattice reflections visualized by selected-area dark field readily enables the distinction between the larger enamel crystals and the smaller underlying dentin crystals (Fig. 6b and c). These a,b + c-axes show enamel crystals to be spatially intimate with dentin crystals; also, enamel crystals are easily recognized within dentin furrows.

Discussion

The mechanism of apatite nucleation in enamel cannot be simply extrapolated by analogy to other vertebrate hard tissues because of its ectodermally derived unique protein matrix which lacks both collagen fibrils and matrix vesicles. Thus, the suggestion has been made that nucleation of enamel crystals is dependent on the presence of mineralized dentin [16–18]. This interpretation is based on the temporal sequence of deposition of crystals formed initially in dentin followed by those in enamel and their observed intimate spatial relationship defining the DEJ. Based on conventional electron microscopy, Reith [16] and Bernard [17] suggested that following the appearance of mineralized dentin, the crystals of enamel arise and grow from the existing dentin crystals. This developmental sequence of dentin and enamel calcification was observed in the present study as follows: (1) nascent sites of mineral deposition within the dentin matrix occur subjacent to the DEJ (Fig. 1a); (2) growth of these mineral deposits extends to the terminal ends of dentin collagen fibrils at the DEJ (Fig. 1b and c); (3) concom-

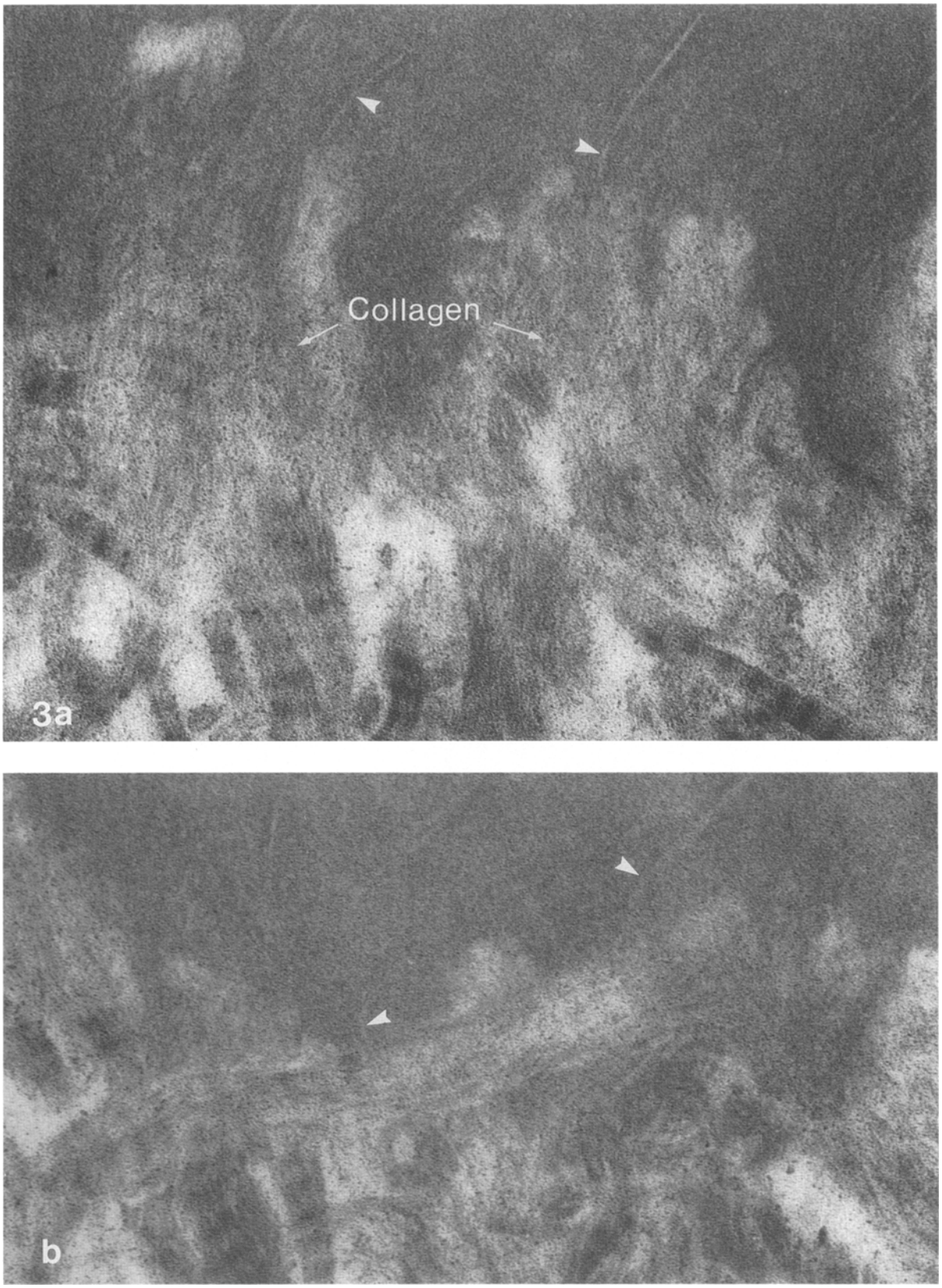

Fig. 3. Prolonged EDTA/stained sections at higher magnification of the DEJ. Terminal ends of frayed dentin collagen fibrils show a reduced banding periodicity and have an expanded, fibrillar appearance. The dense enamel matrix is marked by lucent areas (arrowheads) formerly occupied by mineral; these lucent areas are found in close association with dentin collagen fibrils both at their terminal ends (**a**) and lateral borders (**b**). The punctate distributions associated with the collagen fibrils are lead stain deposits. Molar, glutaraldehyde perfused. ×122,200.

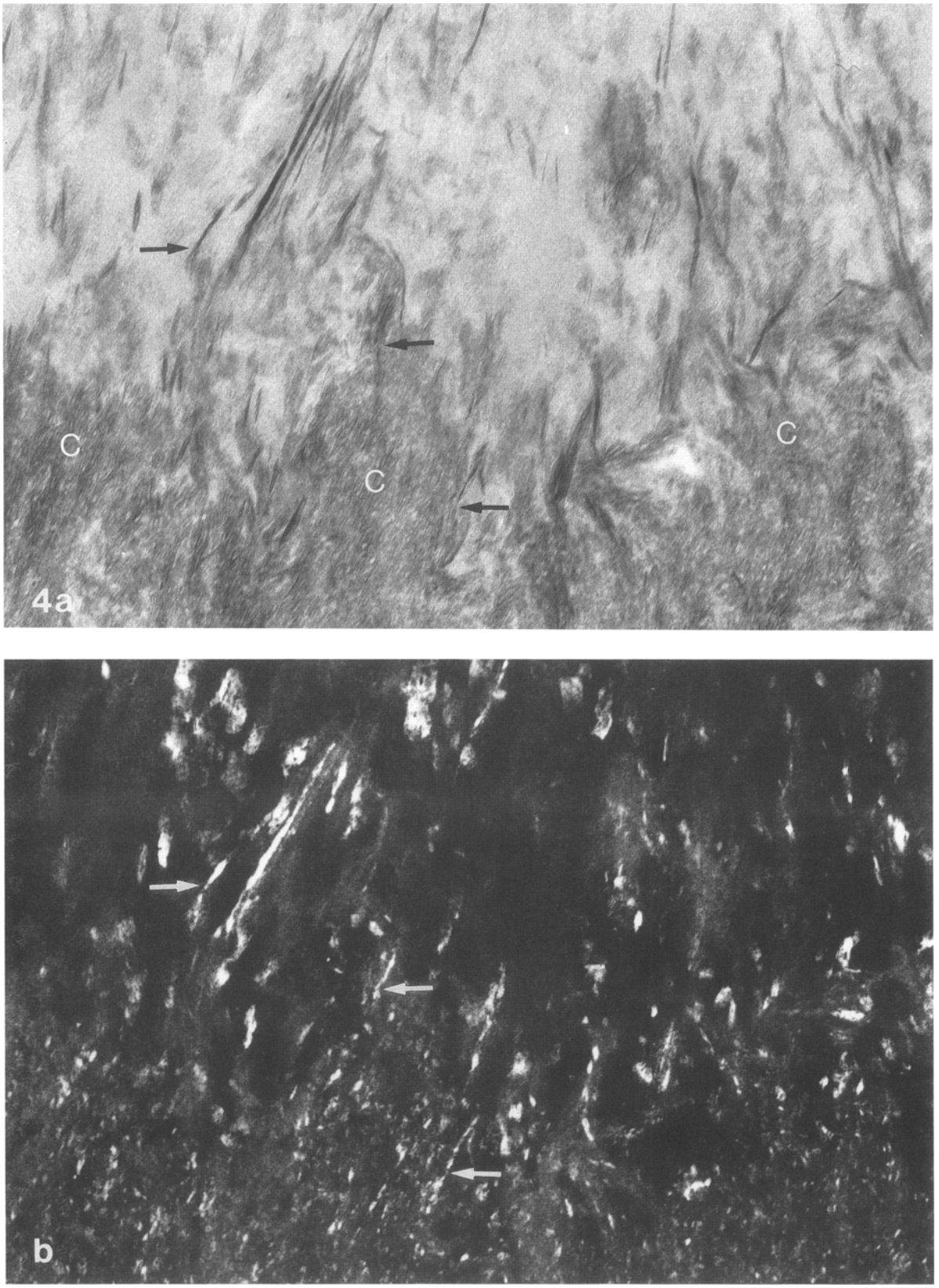

Fig. 4. Companion bright field **(a)** and selected-area dark field **(b)** images of the same area of the DEJ at a stage similar to Figure 1e and f and Figure 3. **(a)** Only the high mass densities of apatite crystals in both the enamel and dentin are visualized. Calcified dentin collagen fibrils (C) project into the enamel matrix. **(b)** A combined axial image illustrates the close association between enamel and dentin apatite crystals. In **(a)** and **(b)** images the corresponding arrows indicate a close spatial relationship between enamel and dentin crystals. Molar, slam frozen/freeze substituted. ×138,000.

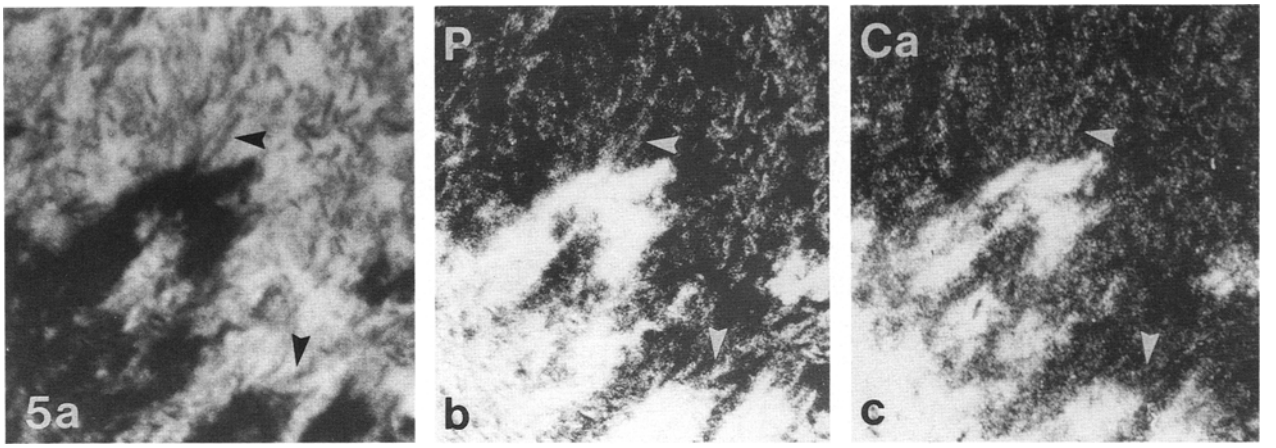

Fig. 5. (a) An electron spectroscopic image (150 eV) of the dentino-enamel junction at an early stage of enamel formation. (b) and (c) Elemental maps of this same area showing the spatial distributions of phosphorus and calcium displayed as white pixels on a black background. Co-localization of P and Ca was not restricted to the apatite domains of calcified dentin collagen and enamel crystals but also present within the enamel sheaths (arrowheads). Molar, slam frozen/freeze substituted. ×45,000.

itant with the calcification of these collagen fibrils is the detectable release of enamel matrix components by ameloblasts (Fig. 1b and c); (4) in the presence of this amorphous enamel matrix the first enamel crystals appear extending from the calcified dentin (Fig. 1d); and (5) these early enamel crystals grow into this matrix to the apical border of the ameloblasts, and with the continued deposition of enamel matrix and mineral, the developing enamel layer thickens (Fig. 1e and f).

Several functional possibilities can be attributed to the close spatial relationship between the dentin and enamel: Dentin may serve as a structural foundation onto which the matrix and crystals of enamel are deposited; this may involve a chemical bonding between the two matrices. Chemical interactions between the two matrices may serve to promote enamel crystal nucleation; in this case the organic and/or inorganic components of dentin may alter the enamel matrix to promote enamel crystal formation. Exposure of dentin crystals directly to the enamel matrix may promote epitaxial growth of enamel crystals. Considering that these interactions may act in concert, one with the other, we propose a working model to summarize the structural events of amelogenesis and to speculate on the spatial relationships between dentin and enamel (Fig. 7). In vertebrate calcified tissues the control of crystal nucleation and growth is mediated through an organic/inorganic interface; such an organic/inorganic relationship may also be extended between the tissues of dentin and enamel at the DEJ. The structural organic components (frayed collagen fibrils, enamel matrix; Figs. 2 and 3), inorganic components (apatite crystals of dentin and enamel; Fig. 4), and elemental co-distributions of P and Ca (Fig. 5) are all shown to have close spatial interrelationships.

Both selected-area dark field imaging for apatite crystal localization (Fig. 4) and high resolution elemental maps of P and Ca localization (Fig. 5) revealed an intimate association between the enamel and dentin domains. These combined results suggest the possibility of a direct or near direct contact between the dentin and enamel crystals; however, a direct crystal continuity between these two matrices remains to be demonstrated. If the initial enamel formation is dependent on the presence of dentin crystals, then it is probable that a consistent crystallographic orientation may exist between these two crystal domains. The determination of the true crystal relationship between dentin and enamel will, however, prove to be a difficult undertaking because of various technical limitations such as spatial overprojection and truncation of small crystals within thin sections [28], limited detection of small crystals having few lattice repeats and potential displacement and fracture of crystals during sectioning. Also, biological apatite is highly susceptible to radiation damage in the electron microscope; this is most apparent when imaging lattice planes.

Given the possibility that apatite crystals within and at the surface of dentin collagen are directly exposed to the enamel matrix (Figs. 4 and 7), then, on exposure to enamel matrix the size restriction imposed on apatite crystals by factors within the dentin matrix is removed. Thus, the dentin crystal in association with the enamel matrix may promote the formation of an enamel crystal, or looked at in a different way, the dentin crystal is allowed to grow into the enamel matrix gradually taking on the char-

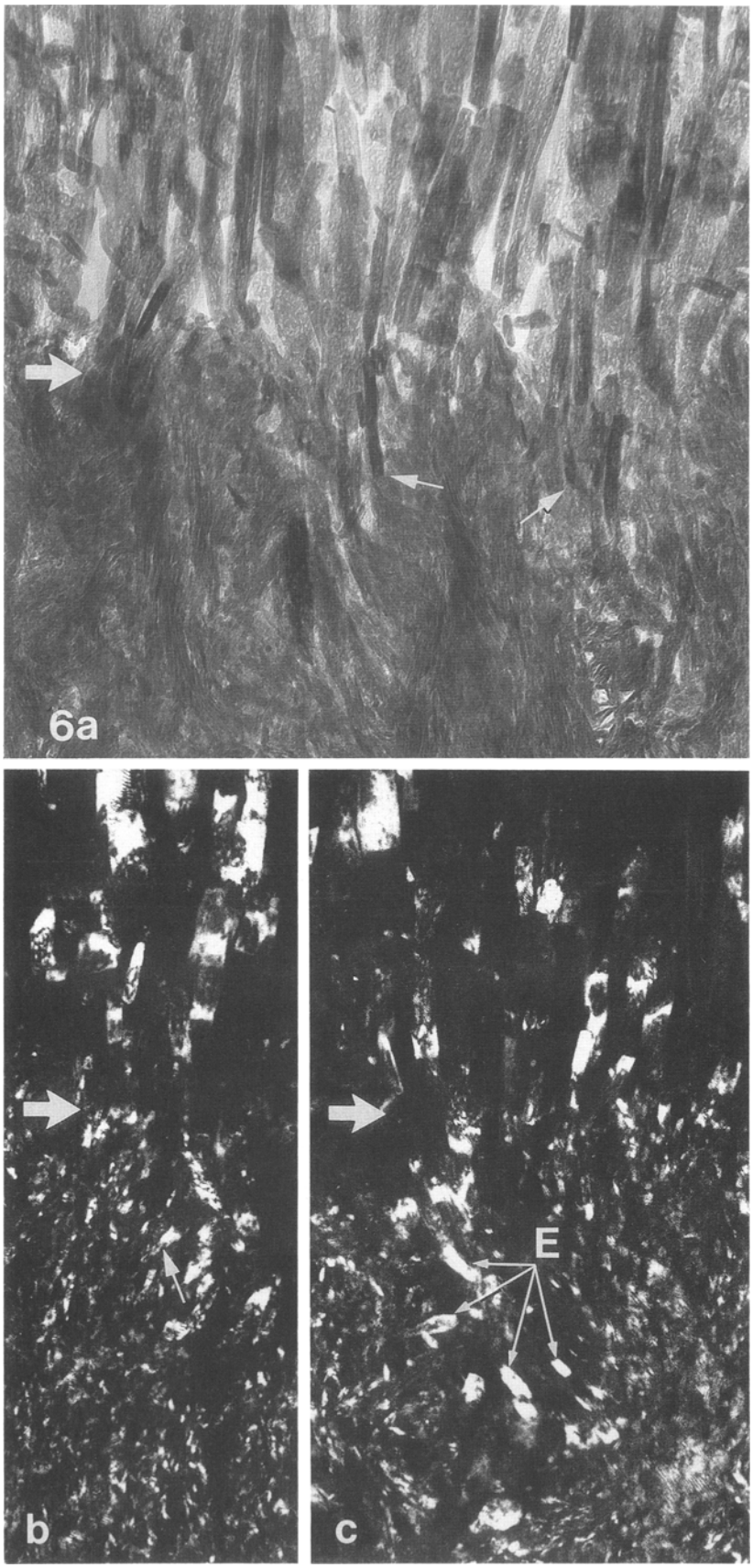

Fig. 6. Dentino-enamel junction. **(a)** A bright field image showing the dentino-enamel junction (large arrow) with enamel crystals abutting the dentin layer and projecting into dentin furrows. Fortuitous planes of section reveal a close spatial relationship between the terminal ends of enamel crystals and the underlying dentin (small arrow). **(b)** A selected-area dark field image illustrating the possible contact between the terminal end of an enamel crystal and a dentin crystal (small arrow). **(c)** Illustrating enamel crystals (E) deep within a dentin furrow. Incisor, air-dried. **(a)** ×96,000; **(b,c)** ×100,000.

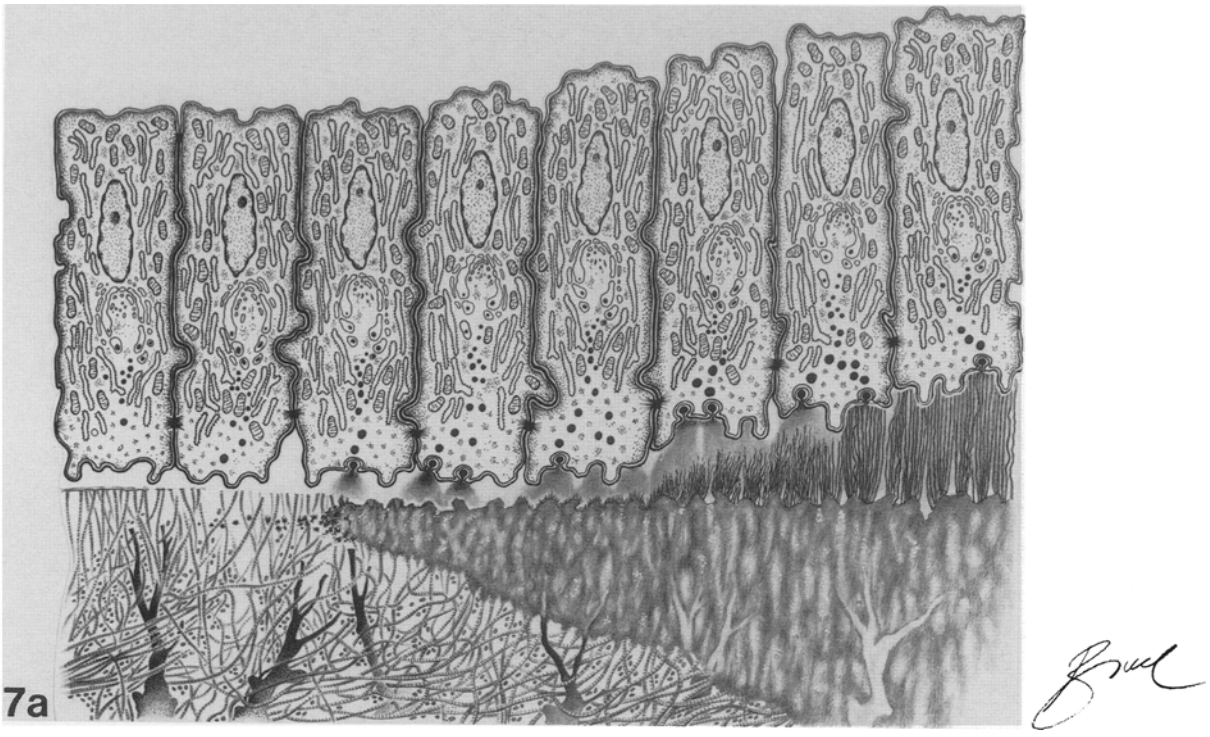

Fig. 7. (a) Schematic representation of the sequential events of dentin and enamel calcification as observed in the rat tooth; this developmental sequence is described in Figure 1. Monolayered ameloblasts are positioned over predentin and calcified dentin matrices; at the point of calcified dentin, the ameloblasts secrete a dense matrix component into which enamel crystals grow from the dentin. Within the predentin, nascent mineral deposits occur subjacent to the DEJ; the lateral growth of these sites spreads to the terminal ends of collagen fibrils at the DEJ. Cellular processes of underlying odontoblasts are shown in dentin and predentin. Continued on page 120.

acteristics of an enamel crystal with further growth. With the exception of enamel crystals, all of the other studied skeletal tissues containing matrix vesicles and collagen (calcified cartilage, bone, dentin, and calcified turkey leg tendon) have basically the same crystal c-axial dimensions of 110–170 Å as determined by selected-area dark field and X-ray diffraction [21, 22]. The factors that regulate this biologically constant crystal size in both fibrillar and extrafibrillar spaces are obviously not present in this unique enamel matrix. Enamel crystals having potentially been epitaxially induced by calcified dentin are then freed of such constraints, allowing them to grow progressively in size with maturation.

Concomitant with the appearance of enamel crystals are their surrounding enamel sheaths which have an intimate association with the surface of dentin collagen (Figs. 2 and 3). High resolution elemental maps revealed the distribution of P and Ca in calcified dentin collagen fibrils to be continuous with the co-localization P and Ca within the enamel sheaths. This presumed organic/inorganic interface between the enamel sheath and the enamel crystal may provide a localized environment for the acquisition of Ca/P and the growth of enamel crystals. Such loci positioned over a calcified dentin matrix would serve as conduits allowing enamel crystal growth to extend from predisposed dentin crystals. Thus, the enamel sheath may act to compartmentalize each of the growing crystals which provides a local environment for their continued growth and organization into rod/interrod enamel. This enamel sheath may, in part, represent the presence of enamelins which have been isolated from the enamel matrix [2] and immunolocalized to enamel crystals [29]. In the present study, the localization of phosphorus distribution within the enamel sheath is corroborated by biochemical evidence for the presence of phosphopeptides [30] and that certain molecular species of enamelins are highly phosphorylated [31]. Isolated and stained enamel components (presumably acidic enamelins) have also been studied by electron diffraction and found to have a β-pleated sheet configuration (as observed for enamel phosphopeptides by Glimcher et al. [32]) and to possess a translational repeat similar to the c-axial 002 d spacing of apatite [33].

The temporal and spatial dependence of enamel crystal formation on calcified dentin serves to integrate enamel calcification with the mineralization

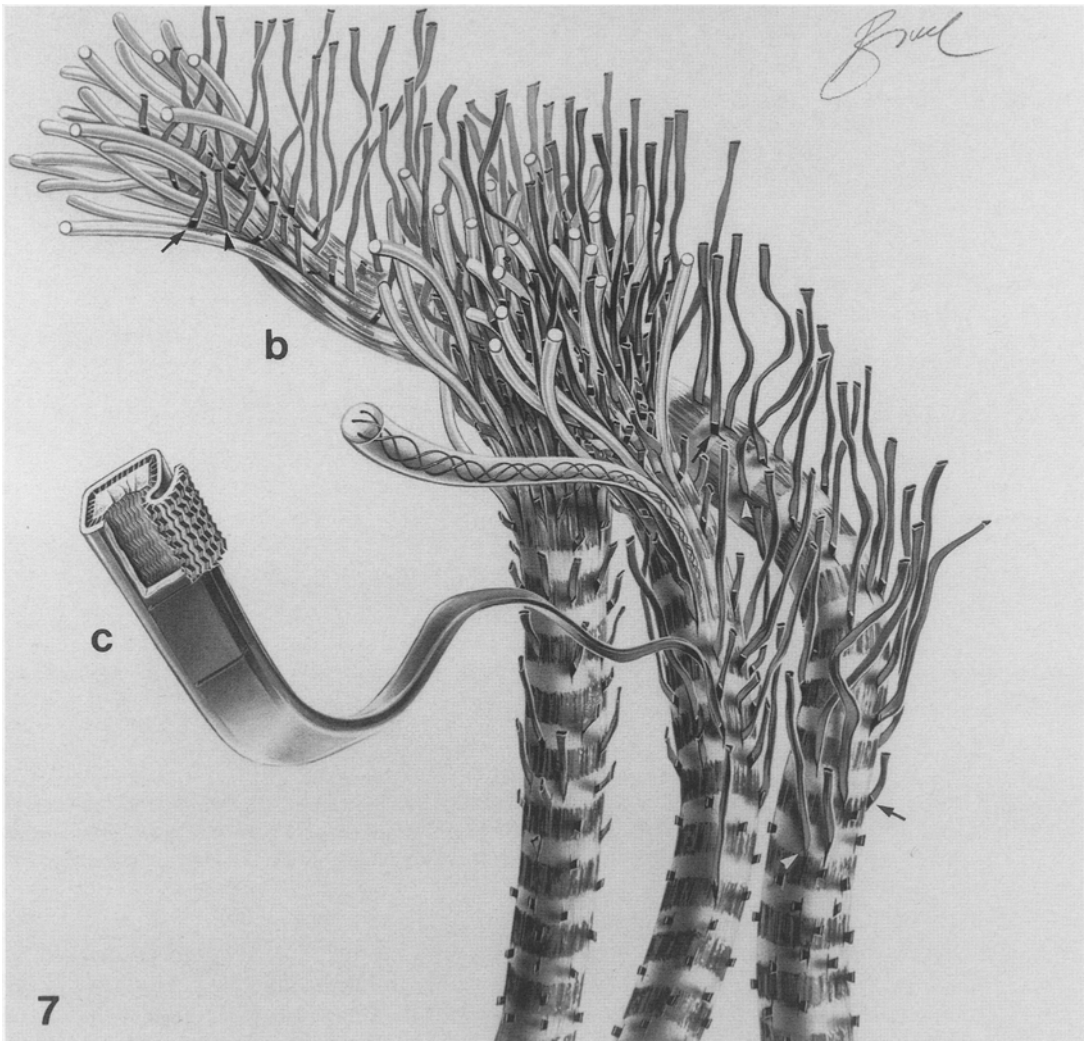

Fig. 7. Continued from page 119. **(b)** An expanded view of the DEJ during the early events of enamel crystal formation. The lateral surfaces and terminal ends of dentin collagen fibrils with associated dentin apatite crystals are directly exposed to the enamel matrix. The collagen molecules are depicted as hollow cylinders (one cylinder is expanded to show the collagen triple helix); enamel sheaths are angular opaque structures containing enamel crystals; dentin crystals are small dense rectangles associated with the dentin collagen fibrils. The ends of the collagen fibrils are frayed providing an intimate relationship with the enamel matrix. At many sites, enamel sheaths and the contained enamel crystals are shown to be directly associated with predisposed dentin crystals (arrows) indicating epitaxial growth of enamel crystals. Also, enamel crystals and sheaths are depicted to be attached to collagen remote from dentin apatite (arrowheads). **(c)** A close-up and cross-sectional view of an enamel crystal and its encapsulating enamel sheath.

sequence of dentin. In skeletal tissues, the first detectable sites of apatite are associated with matrix vesicles, as demonstrated by selected-area dark field imaging in calcifying growth plates [20]. In cartilage and those calcifying tissues containing type I collagen (bone, dentin, turkey leg tendon), the spread of mineral centers about matrix vesicles; in turn, these loci expand and subsequently the matrix calcifies. This observed fidelity of matrix vesicle-mediated calcification may also be extended to enamel; in this case matrix vesicles of dentin are the initial site of apatite formation [34]. This is followed by the spread of mineral throughout dentin, and as shown in this study, early enamel apatite crystals are formed in intimate association with dentin crystals located at the terminal ends of dentin collagen fibrils. The mechanism by which the matrix vesicle-associated mineral induces crystal deposition into the surrounding extravesicular environment may be similar to how calcified dentin promotes the initial

enamel crystal formation. In this fashion, once apatite mineral has been formed, it may influence its local environment in such a way as to promote the nucleation and growth of adjacent crystals.

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