A MORPHOLOGICAL STUDY OF THE FORMATION OF THE DENTINO-ENAMEL JUNCTION
IN THE RAT MOLAR: THE ROLE OF DENTIN IN ENAMEL FORMATION.

By

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ABSTRACT

This thesis is a morphological study detailing the initial formation of the dentino-enamel junction (DEJ) in the rat molar. It was undertaken to determine how enamel apatite crystals are initiated during tooth development. The DEJ was investigated along its developmental gradient so that both the spatial and temporal sequences of dentin and enamel mineralization could be observed. For this investigation both conventional and selected-area dark-field electron microscopy were applied. The results of this study indicate that enamel crystals arise by epitaxial growth from preformed dentin crystals. This is supported by the temporal relationship beween dentin and enamel where dentin mineralization preceeds enamel mineralization and the intimate spatial relationship between the apatite crystals of dentin and enamel at both the newly formed and the more mature dentino-enamel junction. These results also suggest that the terminal portions of collagen fibrils in the pre-dentin matrix undergo some alteration prior to mineralization which may be related to their function in presenting dentin apatite to the enamel matrix. The interrelatonship between both the inorganic and organic matrices of dentin and enamel are presented in a working model and discussed in light of the mechanisms by which mineralized dentin may promote the formation of enamel crystals.

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ABBREVIATIONS

BF   bright field

BL   basal lamina

BM   basement membrane

Ca2+   calcium

DAJ   dentino-ameloblast junction

DEJ   dentino-enamel junction

ECM   extracellular matrix

EDTA   ethylenediaminetetra-acetic acid

EM   electron microscopy

GAG   glycosaminoglycan

hrs   hours

LC   lead citrate

LM   light microscopy

min   minutes

mls   millilitres

nm   nanometers

PTA   phosphotungstic acid

P   phosphate

RER   rough endoplasmic reticulum

SADF   selected-area dark field

TEM   transmission electron microscopy

UA   uranyl acetate

μm   micrometers

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ACKNOWLEDGEMENTS

HISTORICAL REVIEW

This thesis deals with the events of mineralization in tooth dentin and enamel in relation to the formation of the dentino-enamel junction (DEJ). At this junction there exists an intimate spatial relationship between these two extracellular matrices (ECMs). This relationship is important because it provides a firm attachment for enamel to dentin enabling the tooth to maintain its structural integrity while involved in mastication. New evidence of this relationship at the DEJ is explored at the electron microscopic level in this thesis, but in order to fully understand how this relationship is formed and what components are involved, a comprehensive review is required. The review presented here provides background information about the cellular interactions leading up to the formation of the DEJ, the biochemistry of the organic components of dentin and enamel, how mineralization is thought to occur, and specific information about apatite crystal.

DENTIN

1) Cellular Development

The cells responsible for elaboration of the dentin matrix are odontoblasts. Their differentiation from mesenchymal cells in the stomodeum is thought to result from genetic predetermination and interaction with the overlying epithelium during odontogenesis (Thesleff and Hurmerinta,1981; Slavkin et al., 1984).

The mesenchymal cells in the maxillary and mandibular arches

that give rise to odontoblasts are collectively referred to as ectomesenchyme. Unlike the surrounding mesenchymal cells, the ectomesenchyme is thought to have arisen from the neural crest. Along the arches these cells form distinct clusters beneath the oral epithelium (Weston 1981). Studies of carbohydrate histochemisty have shown that the presence of N-acetyl-D-glucosamine in the basement membrane (BM) causes the condensation of the ectomesenchymal cells (Lau and Ruch, 1983). The BM also has been implicated as having a role in the differentiation of the ectomesenchymal cells into pre-odontoblasts (Ruch, 1984). A recent study by Blottner and Linder (1987) using peroxidase conjugated to different lectins, demonstrated progressive stages of odontogenesis based on the spatial and temporal expression of three sugar residues associated with the BM.

With in vitro studies Kollar and Baird (1970) grew molar ectoderm on incisor mesenchyme and vice versa observing in both cases that the mesenchyme controlled both the morpho- and cytodifferentiation of the ectoderm to form the mesemchymal type of tooth germ. This inductive control by mesenchyme is not limited to oral ectoderm, as the mesenchyme can form tooth germs when associated with ectoderm from other sites on the body. While the mesenchyme seems to orchestrate the formation of tooth germs, it cannot differentiate without an associated ectoderm (Thesleff and Hurmerinta, 1981).

Electron microscopic studies have demonstrated a prominent BM between the epithelial and the mesenchymal cells (Bernard, 1972; Slavkin and Bringas, 1976). Biochemical studies have characterized this dental BM to be composed of type IV collagen, heparin sulphate,

laminin, sulphated proteoglycans and fibronectin similar to that of other BM (Kefalides, 1973; Martinez-Hernandez et al., 1981; Birembaut et al., 1983). During the time when mesenchymal cells organize along the BM, its fibronectin content increases (Thesleff et al., 1981). This finding supports theories for the role of fibronectin in attaching mesenchymal cells to the BM whereby they undergo a polarization of cellular organelles and begin to increase the amount of secretory activity. Mesenchymal cells that reach this stage of differentiation in the tooth germ are referred to as pre-odontoblasts. Histologically, these cells are organized into a layer of postmitotic, low columnar cells with basally located nuclei. Each cell has an increased amount of rough endoplasmic reticulum (RER) which aligns parallel to the long axis of the cell, a centrally located Golgi apparatus and numerous free ribosomes and mitochondria (Reith 1968). Mesenchyme cytodifferentiation is also associated with an increased synthesis and release of sulphated glycosaminoglycans (GAGS) and glycoproteins into the ECM apical to pre-odontoblasts (Thesleff and Hurmerinta 1981).

Trans-filter studies are used to demonstrate the requirement of a close proximity between pre-odontoblast cells and the BM of the enamel epithelium for odontoblast differentiation to occur. In these studies pre-odontoblast and enamel epithelial cells are enzymatically separated and placed on opposite sides of filter paper, and allowed to continue to differentiate (Thesleff and Hurmerinta, 1981). Only the mesenchymal cells whose apical cell processes are closely associated with the BM of the epithelium differentiate into pre-odontoblasts. Experiments where the BM of developing tooth germs were disrupted with

vitamin A or tunicamycin, the mesenchyme did not differentiate (Hurmerinta et al., 1980; Thesleff and Pratt, 1980).

The pre-odontoblast, after initial differentiation, begins to secrete a predentin matrix which is composed of collagen types I, III, IV, V, proteoglycans, glycoproteins, serum proteins and matrix vesicles (Linde, 1985). During this time the pre-odontoblast cell continues to differentiate becoming taller and being pushed further away from the BM. The only portion of the cell that remains in close proximity to the BM is its apical cell process.

2) Predentin/Dentin Organic Matrix

Predentin and dentin differ with respect to the absence or presence of a mineral component. Other differences between the organic matrices of predentin and dentin are observed biochemically as changes in the types and concentrations of non-collagenous proteins (Butler 1984a). Another difference observed at the electron microscopic (EM) level is the presence or absence of matrix vesicles; these vesicles are only present in the predentin prior to its initial mineralization (Bernard, 1972; Katchburian, 1973). Once the initial layer of dentin is formed, matrix vesicles are not observed in either the predentin or the dentin.

A) Collagen

A number of types of collagen have been identified in the pre-dentin matrix, they include: type I, III, IV, and V, but only collagen types I and V are found in dentin (Butler 1984b). Type I is the most abundant protein, making up 80-90 % of both matrices (Linde, 1985). It appears randomly arranged as individual fibrils throughout the matrices or as organized bundles, referred to as von Korff fibres,

extending from between the pre-odontoblast cell processes to the BM in pre-dentin. Both groups of fibrils show the characteristic 64 nm repeated banding pattern by EM analysis (Reith, 1968; Wittaker et al., 1972; Larsson and Bloom, 1973; Wigglesworth et al., 1986).

The formation of collagen type I from odontoblast cells has been studied using tritiated proline autoradiography (Weinstock and Leblond, 1974). Each collagen molecule is made up of a triple helix; these molecules are organized in a staggered fashion and are held together by crosslinks. At the ends of the molecule there is a space which, because of the regular stagger of molecules, lines up laterally in the fibril creating the regular cross-banding pattern seen at the EM level when stained with heavy metals (Hodge and Petruska, 1963). The lateral spaces of the collagen fibril are referred to as the gap zones and the area between them is referred to as the overlap zone.

There has been much debate over the existence of von Korff fibres in the pre-dentin matrix; Ten Cate et al. (1970) denied their existence because the fibres that appear at the light microscopic (LM) level when impregnated with silver correspond to ground substance and not collagen when viewed at the EM level. This controversy may lie in the definition of a von Korff fibre. Reith (1968), Wittaker et al. (1972) and Wigglesworth et al. (1986) define these fibres based at the EM level as organized bundles of collagen fibrils, whereas Ten Cate (1970) is defining them as argentophilic fibres observed at the light microscopic (LM) level. These fibres may have a role in orienting the enamel mineralization because they run perpendicular to the BM in the pre-dentin matrix (Wigglesworth et al., 1986).

The von Korff fibres can be 100-250 nm in diameter and stretch

the width of predentin whereas individual collagen fibrils within dentin are 25-50 nm (Johansen, 1964). During mineralization dentin crystals seem to radiate along both these organizations of collagen type I fibrils - their proposed function will be discussed later. Quantitative information on changes in collagen fibril morphology has not been presented at this time.

Collagen type V has been identified in predentin and dentin. It is located in these two matrices near or associated with cellular components; however, its function is not known (Butler, 1984b). The presence of collagen type III in the predentin matrix is somewhat controversial. Garant and Cho (1985) have suggested that this collagen makes up the reticular network of aperiodic fibrils present beneath the BM. Collagen type III may have a role in the inhibition of mineralization as it is found in large amounts in connective tissue that does not mineralize, and in the poorly mineralized dentin of patients with osteogenesis imperfecta (Butler, 1984b; Sauk et al., 1980). Normal mineralization only begins after the disruption and removal of the BM and its associated components. An immunolabelling study, however, has shown that collagen type III is not present in either pre-dentin or dentin (Cournil et al., 1979). Those who support the presence of collagen type III in predentin dismiss this study as not being sensitive enough, thus leaving the controversy unresolved (Garant and Cho, 1985).

Collagen type IV has been demonstrated as part of the BM between the pre-ameloblast cells and the pre-dentin matrix (Thesleff et al., 1981). In the BM this collagen forms a structural backbone upon which the other components attach (Timpl et al., 1981). Collagen

type IV present in isolated fractions of pre-dentin disappears after the BM has been disrupted and removed (Butler, 1984b).

B) Non-collagenous proteins

The non-collagenous proteins are a heterogeneous group of molecules that make up the amorphous background material between collagen fibrils. Their identities and functions have been determined by histochemical and biochemical studies. They appear to be similar to those found in the organic matrix of bone (Linde, 1984).

The major groups of non-collagenous proteins include: proteoglycans, y-carboxyglutamate-containing proteins, phosphoproteins, acidic glycoproteins and serum proteins (Butler, 1984a; Linde, 1984). During the mineralization of dentin, these groups have different roles and, as expected, are present in various concentrations and locations.

The proteoglycans of dentin are molecules containing a protein core with one or two GAG chains attached to it, they can be found in the interfibrillar regions of both predentin and dentin (Takagi et al., 1981). Extraction and biochemical analysis of these molecules show that chondroitin-4-sulphate was the major GAG associated with the protein core. Other GAGs present in less amounts include chondroitin-6-sulphate, dermatan sulphate, heparin sulphate and hyaluronic acid (Jones and Leaver, 1974; Rahemtulla et al., 1984). A recent experiment using a high iron diamine EM technique, which specifically demonsrates sulphated proteoglycans, and enzymatic digestion confirms earlier reports of chondroitin sulphate in dentin as well as being associated with the BM prior to dentin formation. Heparin sulphate was also associated with the BM and may have some

role in the cytodifferentiation of odontoblasts (Kugaya an Furuhasi, 1987).

Electron microscopic autoradiography with labelled sulphate ions demonstrated two metabolically different pools of proteoglycans, the first is in the pre-dentin matrix and the other is secreted just in advance of mineralization in dentin (Sundstrom 1971). At the time of mineralization the amount of sulphated glycoproteins decreases, which suggests that these proteoglycans and GAGs have a role in the regulation of mineralization (Linde, 1985). Another role may be in regulating collagen fibril formation and in binding fibrils together (Obrink, 1973).

The y-carboxyglutamic acid containing proteins are small - 47 to 53 amino acid residues. One of these proteins was first isolated by Linde et al. (1982) in dentin, but had previously been purified from bone and named osteocalcin (Price et al., 1976). Recent immunolabeling experiments show that this calcium binding protein does not play a role in initial mineralization because matrix vesicles are not labelled with anti-osteocalcin. Labelled RER and odontoblast processes suggest that it is synthesized by odontoblasts and accumulates in dentin after mineralizaton occurs (de Vries et al., 1988). Its function is unknown.

Another group of non-collagenous proteins are the phosphoproteins. The major amino acids of the phosphoproteins are serine and aspartate which are highly phosphorylated (Dimuzio and Veis, 1978; Richardson et al., 1978). These phosphoproteins, also referred to as phosphoryns, are only found in the dentin matrix, having been secreted at the mineralization front (MacDougall et al.,

1983). Their importance in the process of mineralization is suggested by their absence in dentinogenesis imperfecta II where the dentin is poorly mineralized (Takagi et al., 1983). Isolated phosphoryns have been shown by rotary shadow electron microscopy to bind Ca2+ in the presence of calcium chloride (Cocking-Johnson et al., 1983). Binding domains on phosphoryns, proposed in a model by Lee et al. (1977) suggest that the phosphoryns are involved in nucleating apatite and perhaps control the size of dentin apatite crystals. A recent study by Lussi et al. (1988) demonstrated that purified phosphoryns free in a calcium and phosphate (CaP) solution inhibited the precipitation of apatite, but when the phosphoryns were covalently attached to agarose beads in the same solution apatite formation was induced. Only low amounts of phosphoryns were required to induce crystal formation if attached to agarose beads. Phosphoryns may have both an inhibitory and an inductive role in dentin mineralization.

The presence of other acidic glycoproteins has been determined, but other than their molecular weights, few studies have purified and characterized them (Butler, 1984a; Linde, 1984, 1985). Serum proteins have also been reported, these include albumin, transferrin, IgG, IgM, and IgA (Thomas and Leaver, 1975). Their function in dentin is unknown at the present time.

C) Matrix Vesicles

EM studies have demonstrated matrix vesicles to be present in the pre-dentin matrix. Such vesicles are found beneath the basal lamina in the amorphous material between collagen fibrils. Katchburian (1977) described them as being 30-300 nm in diameter with a limiting trilaminar plasma membrane 7-8 nm wide. They vary in shape

and in the electron density of their constituents and are thought to be formed by budding off from odontoblast processes (Slavkin et al., 1972; Siska and Provenza, 1972). Serial sections and freeze fractures of the pre-dentin matrix have demonstrated that the matrix vesicles are distinct from odontoblast processes even though they appear to have similar contents (Katchburian, 1973, Katchburian and Severs, 1982).

Matrix vesicles are present in the pre-dentin matrix from the point where the mesenchyme become pre-odontoblasts until the matrix is mineralized (Bernard, 1972; Larsson and Bloom, 1973). Within this area Slavkin et al. (1972) recognized distinct types of matrix vesicles based upon their size and electron density. Katchburian and Sever (1982) also recognized a heterogeneity between matrix vesicles when they compared the number of intramembrane protein particles of each vesicle revealed by freeze fracture replication.

A function proposed to be associated with one group of matrix vesicles is the initiation of dentin mineralization. This was suggested initially because electron-dense material similar in shape to the apatite crystals observed in dentin was found in some vesicles (Slavkin et al., 1972; Katchburian, 1973; Bernard, 1973; Eisenman and Glick, 1972). Other evidence for this function shows that isolated matrix vesicles from pre-dentin use ATP to concentrate calcium (Granston, 1984). As there are vesicles which do not contain matrix vesicles not all matrix vesicles are thought to be involved in the initiation of mineralization in pre-dentin (Slavkin et.al., 1972).

Another function of matrix vesicles is in the transport of enzymes, acting as extracellular lysosomes (Slavkin et al., 1972;

Katchburian, 1973). Evidence demonstrating the presence of acid phosphatase within these vesicles supports this function (Larsson, 1973). Matrix vesicles may also have a role in the transportation of structural molecules into the matrix from the odontoblasts, and they may provide a means of communication between the pre-odontoblast and the pre-secretary ameloblast prior to calcification (Slavkin et al., 1972). These last two functions are more difficult to substantiate.

The presence of matrix vesicles is not limited to predentin, they are also found in cartilage, bone, and calcifying extra-skeletal tissues where they appear primarily to be involved in the initiation of mineralizing a collagenous extracellular matrix (Bonucci, 1984). Arsenault and Hunziker (1988) were the first to specifically determine the presence of apatite within matrix vesicles. This was acheived by using selected-area dark field (SADF) imaging on epiphyseal cartilage preserved by high pressure freezing.

3) Mineralization

EM studies have described the process of mineralization of pre-dentin as beginning in membrane-bounded matrix vesicles containing electron-dense crystal-like structures. The crystals increase in number within the vesicle and then exceeds past the vesicle membrane, growing epitaxially through the afibrillar ground substance. These radiating crystals appear as islands or loci of electron density in unstained sections (Hayashi, 1984). Crystal growth continues through the ground substance to collagen fibrils which becomes impregnated with the mineral. As mineralization progresses, the separate loci coalesce and form a continuous layer described as mantle dentin (Bernard, 1972; Siska and Provenza 1972; Hayashi, 1984; Bocchieri et

al., 1985).

Several mechanisms have been proposed for the role of matrix vesicles in initiating mineralization in dentin. Katchburian (1973) proposed that these vesicles originate from odontoblasts either being synthesized in their cytoplasm and then exocytosed or by budding off odontoblast cell processes. It was assumed that the vesicles contained Ca and P ions in similar concentrations to that found in odontoblasts, however, while in the predentin the plasma membrane of a vesicle undergoes a change in its permeability because it is no longer associated with a cell and therefore is not renewed. The change in membrane permeability results in the creation of a super-saturated ionic condition that precipitates apatite crystal. Another author suggests that the matrix vesicles concentrate ions by ATP/CA2+ pumps detected in membranes of isolated vesicles (Granstrom, 1984). Phosphate head groups of membrane lipids may be responsible for binding Ca ions concentrated within the vesicles and precipitating apatite crystals which appear to form on their inner membrane surface (Eanes and Hailer, 1985).

There is an increase in crystal number and size within the vesicles until the vesicle can no longer contain them, it bursts and crystal growth extends into the surrounding predentin. Matrix vesicles do not have a role in subsequent mineralization as they are not observed in predentin after mantle dentin is formed (Eisenman and Glick, 1972). Continued mineralization is thought to be due to a high concentration of Ca and P, both free and protein-bound in the predentin matrix (Munhoz and Leblond, 1974; Butler, 1984).

The calcium concentration of the dentin matrix is controlled

by the odontoblasts. Studies using pyroantimonate show that calcium is taken up by odontoblasts from laterally and basally located capillaries and deposited apically in the extracellular matrix (Appleton and Morris, 1979). The pathway of calcium through the odontoblast cells is confirmed by the presence of tight junctions between cells which lanthanum, an ion similar in size to calcium, cannot traverse (Bishop, 1985). During continued mineralization, calcium ions are also supplied by the supporting layers of ameloblasts - the stratum intermedia and stellate reticulum.

The presence of phosphate in the dentin matrix associated with phosphoryns has been discussed in relation to its proposed role in apatite formation. Not much is known about the mechanism of phosphate transportation into the pre-dentin matrix but x-ray microanalysis and micro-chemical analysis has demonstrated its presence in relatively high concentrations (Höhling and Fromme, 1984).

Initial crystals formed within and around matrix vesicles appear to be needle-like with an average width of 2.6 nm. As mineralization progresses, crystals are described as more plate-like and measure about 5-6 nm in width (Hayashi, 1983, and Frank, 1980). This increase in width is thought to result from the fusion of the initial needle-like crystals (Hayashi, 1984). Fusion has also been implicated in the preferential mineralization of collagen fibrils.

The initial mineralization of collagen was thought only to occur in gap zones of collagen fibrils (Glimcher, 1959). It has since been demonstrated with conventional EM and SADF imaging that mineralization can occur in both the gap and the overlap zones, although· to a lesser extent in the latter (Arsenault, 1988; 1989;

Hohling et al., 1971). The role of collagen in mineralization may be in providing an environment which traps calcium and phosphate ions giving an ion pathway for crystal growth to follow.

4) <u>Dentin Crystals</u>

Electron diffraction and x-ray diffraction patterns show dentin crystals to be the insoluble calcium phosphate salt, hydroxyapatite (Trautz, 1966; Posner and Tannenbaum, 1984). Chemical analysis of the crystals show that they are not pure hydroxyapatite, but contain carbonate, water and trace inorganic constituents (Petersson et al., 1978). These impurities alter the molar ratio of calcium to phosphate which is approximately 1.67 in mature dentin and slightly less in forming dentin (Posner and Tannenbaum, 1984).

Dentin crystals, like other crystals in mineralized tissues, have a unit cell with the chemical formula $Ca_{10}(PO_4)_6(OH)_2$ and dimensions deliniated by x-ray and neutron diffraction of 0.942 nm in the a- and b-axis and 0.688 nm in the c-axis (Glas, 1962). These unit cells are parallelpiped in shape and act as a template for the atomic arrangement of the whole crystal. They line up so that the c-axis is the long axis of the crystal and the a-axis is the thickness. Because these unit cells are arranged in an equal, repeated pattern they can be used to derive both the overall size and orientation of crystals in dentin.

A number of studies have measured the dimensions of mature dentin crystals, which are tightly regulated, to be 20 to 35 nm long and 5 - 6 nm thick and wide (Jensen, and Möller, 1948; Trautz et al., 1953; Frank, 1980). There have been few studies to delineate the organization of crystals within dentin.

ENAMEL

Enamel is unlike any other mammalian mineralized tissue because it is formed by ectodermally derived cells, and does not contain collagen, or matrix vesicles and is more highly mineralized.

1) Cellular Development

So far only the embryonic epithelial/mesenchymal interactions leading to dentin mineralization have been reviewed. This interaction is also required for the oral epithelium of the stomodeal membrane to become secretory ameloblasts and form enamel.

The underlying mesenchyme is responsible for inducing the invagination of the stomodeal ectoderm and its eventual morpho-differentiation forming the enamel organ of the tooth germ. Morpho-differentiation results from an increased mitotic activity of the cells of the stratum germinitivum layer. Once morphological differentiation has ceased, the tissue organization resembles the tooth shape described histogically as the bell stage (Reith, 1967). As described in the dentin review, the inner enamel epithelium, which rests upon a BM, induces the mesenchyme cells on the other side to differentiate into odontoblasts and begin to secrete the collagenous pre-dentin matrix. The BM has been implicated in the cyto-differentiation of odontoblasts, but its association with the predentin matrix induces polarity in the inner enamel epithelium. This polarization results in a distally located nucleus, centrally located Golgi apparatus, an increase of mitochondria located apically and distally, and many cisternae of RER running parallel to the long

axis of these cells (Kallenbach, 1972). The pre-dentin matrix contains collagenases that may degrade the BM (Sorgente et al., 1977) which is then removed by the pre-secretory ameloblast cells. EM evidence of pinocytotic vesicles along the apical border of ameloblasts suggests their role in the endocytosis of the broken down BM (Bernard, 1972; Kallenbach, 1976). Following the disruption of the BM, proximal processes of the ameloblasts are observed in direct contact with odontoblast processes; this event may induce the ameloblast to begin the removal of the BM (Slavkin and Bringas, 1976). Contact between these two cells and the removal of the BM are concurrent with the appearance of electron-dense secretory vesicles near the apical cell border within the pre-secretory ameloblast. These secretory vesicles are not released until dentin mineralization reaches the dentino-ameloblast junction (DAJ) (Bernard, 1972). When mineralization reaches this point, enamel matrix is released by exocytosis described as modified merocrine secretion (Simmelink, 1982; Nanci et al., 1987). The electron-dense enamel matrix bathes mineralized collagen fibrils of dentin (Bernard 1972). Soon after the matrix is released it mineralizes, without the aid of matrix vesicles. The initial plate-like enamel crystals are observed to be formed at the dentino-enamel junction and grow toward the ameloblast cell (Reith 1967). It has been suggested by Bernard (1972) that enamel crystals are just extensions of dentin crystals.

2) <u>Enamel Proteins</u>

The newly formed enamel matrix has an organic component and a mineral component. The major part of the organic component is the enamel proteins which make up 20-30% of initial matrix. These

proteins are characterized by their amino acid composition, molecular weight, and on their biochemical properties, it is now agreed that there are two groups of enamel proteins - the amelogenins and enamelins.

The amelogenins are the smaller molecular weight proteins ranging from 5-40 kd, but they make up 80-90% of the organic matrix in initial enamel (Termine et al., 1980). Isolation of this group of proteins with 6M guanidine HCl and analysis of their amino acid content shows them to be rich in proline with lesser amounts of glutamic acid, leucine, and histidine (Eastoe, 1979, Slavkin et al., 1982). It has been suggested that these proteins make up a large portion of the stippled material into which the enamel crystals grow during the initial mineralization of enamel. The amelogenins are not thought, however, to be closely associated with the crystals because they are easily extracted leaving the crystals intact (Termine et al., 1979; Lyaruu et al., 1982). Immunolabelling of the enamel matrix has shown that the amelogenins occupy the matrix between enamel crystals, which suggests they have a space filling role in the enamel matrix (Hayashi et al., 1986).

In freeze fracture of enamel the amelogenins have a globular appearance which is dispersed throughout the matrix between crystals. Their appearance was similar to other non-structural proteins like albumin and ovalbumin after incubation with extracted enamel crystals and freeze fracture (Bai and Warshawsky, 1985). Other evidence for their space filling role comes from studies that correlate changes in the protein content of enamel as it matures. It appears that proteases are secreted by the ameloblasts that selectively breakdown

amelogenin proteins which are then selectivly removed (Overall and Limeback, 1988). This loss of amelogenins corresponds with an increase in mineralization. These changes of the amelogenins are well documented (Termine et al., 1979, Robinson et al., 1977, 1983, Sasaki et al., 1982, Robinson and Kirkham, 1985, Bai and Warshawsky, 1985). Studies comparing amelogenins between species show this group of proteins is conserved (Slavkin et al., 1982; Robinson and Kirkham, 1985).

The other major group of proteins in the enamel matrix are the enamelins. These are large molecular weight acidic glycoproteins 60-75 kd that are antigenically distinct from amelogenins (Rosenbloom et al., 1986). This group of proteins is thought to be initimately associated with enamel crystals because they can only be extracted after demineralization of the enamel with EDTA (Termine et al., 1979). Analysis of the amino acid composition of the enamelins shows them to be rich in aspartic acid, serine, glutamic acid, and glycine. Confirmation of contact between the enamelins and enamel crystals was demonstrated by antigenic labelling of enamel crystals with anti-enamelin (Hayashi et al., 1986). The enamelins are thought to form an enamel sheath that can be observed electron microscopically in demineralized enamel. This sheath stretches from the dentinoenamel junction (DEJ) to the ameloblast cell surface in all stages of enamel mineralization (Jessen, 1968; Gustausen and Silness, 1969; Yanagisawa and Nylen, 1979; Arsenault and Robinson, 1989).

It is believed that these proteins have a function in mineralization. An early x-ray diffraction study showed that the demineralized enamel matrix gave a cross beta diffraction pattern

suggesting a structural component to this matrix (Glimcher et al., 1961). A more recent study by Traub et al. (1985) confirmed that the enamelins are responsible for a beta-pleated sheet conformation closely associated with enamel crystals. This study also showed that the enamel sheath after demineralization could be remineralized by being incubated in a calcium chloride bath. This suggested that the sheath may have a role in the nucleation of enamel hydroxyapatite crystals. Further evidence for this is presented in this study by Traub et. al. (1985), as the phosphate backbone of the proposed sheath structure corresponds to the unit cell dimensions of hydroxyapatite. Electron spectroscopic image analysis also demonstrates that the enamelin sheath is involved in the co-localization of calcium and phosphate thereby playing a role in the growth of enamel crystals (Arsenault and Robinson, 1989).

Unlike the amelogenin group of proteins, the enamelin proteins remain relatively constant throughout enamel maturation, which are the major protein group in fully formed enamel (Termine et al., 1980). A recent study by Menanteau et al. (1988) suggests a more complicated picture for enamelin. This study demonstrates a heterogeneity within enamelins based on their glycosylation, electrophoretic behaviour and stainability suggesting that due to protein changes may be due to a degradation of enamelin during the maturation of enamel.

3) <u>Mineralization/Maturation</u>

As previously stated enamel mineralization is presumed to occur without the aid of the matrix vesicle because they have not been observed in initial enamel formation (Bernard, 1972). It has been suggested, based on EM analysis, that the enamel crystals are derived

from crystals of the forming dentin matrix which it borders (Bernard, 1972). This point is emphasized because enamel crystals appear in the enamel matrix soon after it is secreted. Analysis of these crystals at the DEJ with conventional EM techniques is difficult because of artifacts of preparation and sectioning (Robinson et al., 1981). The question concerning the origin of enamel crystals was addressed recently using SADF imaging, this study suggests a continuity between dentin and enamel crystal populations (Arsenault and Robinson, 1989).

In addition to forming the organic matrix, ameloblasts are involved in mineralization of enamel by sequestering Ca2+, providing an enclosed environment, and in controlling the organization of crystals within the enamel matrix. Calcium ions enter the enamel matrix from blood vessels in the statum intermedium and the stellate reticulum through ameloblasts (Munhoz and Leblond, 1974). The mechanism of Ca2+ release by ameloblasts into the enamel matrix may be by both secretory granules and Ca/ATPase activity observed at the apical end of ameloblasts (Chen et al., 1986; Sasaki and Garant, 1987). Ameloblasts form a tighly bound layer of cells, freeze fracture and conventional EM have demonstrated tight junctions, desmosomes, and gap junctions between each cell (Sasaki et al., 1984). This layer, like the odontoblast layer, provides a localized environment of concentrated CaP ions, but because of cell movement and cell shape it also controls the organization of enamel crystals within the matrix (Osborn, 1970; Warshawsky, 1987).

Morphological studies have shown that secretory ameloblasts undergo differentiation during enamel formation, which divides it into

three stages. In the first stage ameloblasts appear as low columnar cells which form crystals that lie perpendicular to the DEJ, this initial layer of enamel is referred as aprismatic enamel (Leblond and Warshawsky, 1979).

The ameloblasts in the second stage become much taller and form an apical projection - the Tomes' process. Enamel matrix is secreted from two areas on the apical surface of the ameloblast: the Tomes' process, which is responsible for forming rod enamel, and the apical membrane at the base of Tomes' process which forms interrod enamel (Johansen, 1964). Interrod surrounds the rod enamel and is continuous with the initial layer of enamel. Scanning EM of the surface of immature enamel with the ameloblast layer removed reveals prisms with a central depression arranged in rows (Greenberg et al., 1983; Warshawsky and Leblond, 1979). Comparative studies have demonstrated the Tomes' process, and therefore the enamel prism shape to be species specific (Greenberg et al., 1983; Warshawsky et al., 1981). Prisms are made up of interrod enamel and in life the Tomes' processes fill the central depression, as the ameloblast receeds with enamel thickening, rod enamel occupies the central space in the interrod prism. There are two sets of rods that run at 60 - 90 degrees to one another depending upon where in this layer they are sampled (Jodaikin et al., 1984). Serial section reconstruction of these sets of rods show them to run through this entire layer of enamel (Warshawsky and Smith, 1970). Rod enamel is composed of bundles of aligned apatite crystals, interrod enamel is also composed of apatite, it runs perpendicular to the rods, but is less organized (Glick and Eisenmann 1973; Warshawsky et al., 1981).

In the last stage of enamel formation the ameloblast loses its Tomes' process and diminishes in height. The ameloblast forms an outer aprismatic layer of enamel which is continuous with the first layer and the interrod enamel of the second layer. These cells are also involved in the maturation of the enamel matrix (Leblond and Warshawsky, 1979; Reith and Boyde, 1981).

Maturation of enamel involves the increase in the thickness and width of enamel crystals and the reduction of protein content. This process occurs continually throughout the formation of enamel, but during the last stage it is accelerated. The ameloblasts are involved in the continuous maturation by secreting proteases into the matrix to break down amelogenins, and to a lesser extent enamelins (Limeback, 1987; Overall and Limeback, 1988; Shimizu et al., 1979), resorbing the organic matrix and maintaining the Ca concentration (Chen et al., 1986; Kallenback, 1976; Munhoz and Leblond, 1974; Sasaki, 1983). Accelerated maturation raises the mineral content from 70% to 96% due to the action of two cycling populations of maturative ameloblasts which rapidly breakdown and remove enamel proteins and increase Ca entry into the matrix (Crenshaw and Takano, 1982; Reith and Boyde, 1981).

During maturation there is an increase in the width and thickness of enamel crystals from 5 nm by 5 nm in initial enamel to 60 nm by 30 nm in the most mature enamel (Nylen et al., 1963). There is, however, considerable variation in enamel crystal measurement in the literature because age, species and portion of enamel examined vary in each study (Grove et al., 1972; Jensen and Möller, 1948; Daculsi and Kerebel, 1978). It has been suggested that enamel crystals become

very large as a result of fusion as well as crystal growth (Daculsi and Kerebel, 1978). The increase in crystal size due to maturation observed in enamel is unlike any other mammalian mineralized tissue which seem to have a restricted crystal size.

4) <u>Enamel Crystals</u>

X-ray diffraction studies have shown that enamel crystals, like dentin crystals, have the unit cell dimensions of apatite (Selvig, 1972). Initial enamel crystals, however, have been described as being poorly crystalline with electron diffraction studies. Electron probe microanalysis of these crystals shows them to have a lower molar Ca/P ratio to other biological apatite (Landis et al., 1988). With increased crystal growth the electron diffraction pattern becomes more distinct, indicating that electron diffraction patterns of initial enamel may appear poorly crystalline due to fewer, smaller crystals at this stage (Grove et al., 1972).

Based on measurements of extracted enamel, it is believed that some enamel crystals extend from the DEJ to the surface of the enamel (Daculsi et al., 1984; Simons, 1972). The shape of the crystals is more difficult to interpret due to limitations in the transmission EM. Enamel crystals were initially interpreted as ribbon-like hexagonal rods (Nylen et al., 1963), but Warshawsky et al. (1987) determined that the appearance of a hexagonal ribbon is a result of overprojection of a parallelpiped shaped crystal. Using stereo-tilts and freeze fracture EM they have demonstrated the enamel crystals to be rectangular ribbons.

INTRODUCTION

Tooth morphogenesis has been described as an epithelial-mesenchymal organ system because progression through its developmental stages occurs as a result of inductive interactions between both the cellular and the extracellular components of epithelially and mesenchymally derived tissues (Slavkin et al., 1977; Thesleff and Hurmerinta, 1981). In vitro studies of this process have shown that there is an interdependency between the oral epithelium which differentiates into enamel secreting ameloblasts and the ectomesenchyme which differentiates into dentin forming odontoblasts (Kollar and Baird, 1970; Ruch, 1984; Thesleff and Pratt, 1980).

Morphological studies which demonstrate the developmental and mineralization gradient of tooth morphogenesis suggest that a direct contact between odontoblasts and ameloblasts which occurs just prior to dentin mineralization is an inductive interaction which triggers mineralization (Katchburian and Burgess, 1977; Slavkin and Bringas, 1976; Slavkin et al., 1977). After this interaction dentin mineralization is initiated in the pre-dentin matrix by matrix vesicles similar to those found in other mineralizing collagenous matrices (Arsenault 1988; Arsenualt et al., 1988; Bonucci, 1984; Katchburian, 1973). Mineralization speads from these matrix vesicles through the collagenous matrix towards the surface which is bordered by ameloblasts. Once mineralization reaches this surface enamel matrix is secreted by ameloblasts onto the dentin. Enamel apatite crystals can be observed within this matrix soon after it is secreted

and they extend from the surface of the dentin toward the ameloblasts (Bernard, 1972; Eisenmann and Glick, 1972; Reith, 1967; Warshawsky, 1985). This stage of tooth morphogenesis, where the enamel crystals are initially formed, is not well understood.

It is well recognized that mammalian mineralization requires not only a super-saturated ionic concentration and a structured organic matrix, but also the presence of mineral which will act as an initiating factor to mineralization. In dentin and other mineralizing collagenous matrices this factor is provided by matrix vesicles. The initiation of enamel mineralization is different from these other mineralizing systems because it lacks both a collagenous matrix and matrix vesicles. The question of how enamel crystals are initiated during enamel mineralization was first addressed by Bernard (1972). He suggested that enamel mineralization was a secondary mineralization process that was dependent upon dentin mineralization. His morphological study of the early events of enamel formation in the rat molar demonstrated that there was a close association between the crystals of dentin and enamel, but he did not show evidence of a direct crystal continuity. The demonstration of a direct continuity between dentin and enamel would be consistent with the hypothesis for the role of dentin as an initiating factor for enamel mineralization.

Although both enamel and dentin have apatite as the inorganic component of their matrices, the sizes and organizational of those crystals are very different. Factors within the fibrillar and extrafibrillar dentin matrix limit the apatite to a small crystal size, but allow for their random ordering. In enamel, the opposite is true as very large crystals are highly organized within rod and

interrod domains. This varied ordering is reflected by the different chemistry of the organic components of these two matrices (Butler, 1984a; Termine et al., 1979). Crystal size can be used as a criterion to distinguish dentin from enamel crystals at the DEJ where these two groups of crystals are associated.

The temporal and spatial interrelationship of dentin and enamel formation was studied at the EM level along the developmental gradient of the DEJ in the rat molar. Morphological aspects of these processes were examined in both aqueously and non-aqueously prepared tissues with bright field and selected-area dark field imaging techniques. A review of the stages of mineralization in dentin and enamel provided confirmation of the events reported by other authors which include: the initiation of dentin mineralization by matrix vesicles, the fact that dentin mineralization precedes enamel mineralization, and that enamel crystals are formed on the surface of dentin and grow toward ameloblasts (Bernard, 1972; Eisenmann and Glick, 1972; Reith, 1967). A novel view of changes that occur to collagen fibrils at the edge of pre-dentin just prior to mineralization suggests that these pre-dentin collagen fibrils have a specific role in enamel mineralization. The presence of an enamel sheath which borders the apatite crystals in enamel is reviewed with specific interest in the sheath's relationship to the surface of dentin at one end and the ameloblast cell membrane at the other. The apatite crystal of dentin is investigated with selected-area dark field to determine if there is an organization within it. Crystal continuity between apatite of dentin and enamel at the DEJ in both early and more mature stages of tooth development. The implications of

these results are discussed in light of a working model of the spatial relationships between dentin and developing enamel. From this research it appears that enamel apatite crystals form as a secondary mineralization process, as there is a intimate spatial relationship between both the organic and inorganic components of dentin and enamel.

MATERIALS AND METHODS

1) Animals

For each experiment, molars were extracted from the mandibles of 3 to 7 day-old, male Spague-Dawley rats. This age range was selected because all developmental stages of enamel and dentin are present. Mandibular molars were used because the early developmental sequences are more easily identified grossly than in the incisor; this was most important for the slam freezing fixation.

2) Methods of Fixation for TEM:

a) Conventional Specimen Preparation

i) Immersion Fixation: Rats were killed by decapitation and their mandibles were excised, split at their symphysis and immersed in 2.3% glutaraldehyde in 0.05 M sodium cacodylate, buffered to 300 mOsm. with 5% sucrose and adjusted to pH 7.3, at 4°C for 2 hrs. Tissues were washed twice for 15 min in sodium cacodylate buffer at room temperature, and then immersed in a 1% buffered solution of osmium tetroxide for 1.5 hrs. The tissue was washed again as above and then dehydrated in a graded series of methanol (50,70,90,100%) for 15 min each, and three changes at 100% before being transferred to acetone (three changes as well).

ii) Perfusion Fixation: Rats anesthetised with sodium pentabarbitol were first perfused intracardially with Ringer's saline which contained an anti-coagulant (0.025% heparin), a muscle relaxant (0.1% procaine) and was adjusted to 300 mOsm with 5% sucrose and pH 7.3. This saline, like all other perfusates used, was warmed to 37°C

and its flow through a cannula was adjusted to 40 mls/min with a pressure of 100 mmHg to approximate physiological conditions. Saline was perfused until all of the blood was cleared, as observed by clear saline flowing from the cut external jugular veins. After clearing the perfusate was switched to 2.3% glutaraldehyde in 0.05 M sodium cacodylate buffered to pH 7.3 and 300 mOsm for 20 min. Mandibles from each rat were excised, split at the symphysis and immersed in the above fixative cooled to 4° C for an additional hour. During this fixation the half mandibles were divided further at the groove delineating the division between the bony cavities containing the molars and the incisor. Sections of the mandible containing two molars were washed in the sodium cacodylate buffer, prepared as mentioned above, two times for 15 min then immersed in a buffered 1% solution of osmium tetroxide for 1.5 hrs. The tissue was then washed and dehydrated as in the immersion fixation.

b) Cryogenic Specimen Preparation

Excised molars were sliced in half and placed in on filter paper with the cut surface facing up. These were in turn placed on a teflon disc and slam frozen on a high purity copper block cooled to liquid helium temperatures (-270° C) (Arsenault et al., 1988). The disc with the frozen specimen attached was placed in liquid nitrogen until transfer to pre-cooled (-85°C) glass vials with 0.5% glutaraldehyde in 100% methanol. In these vials tissues were freeze-substituted for 48 hrs at -85° C then the temperature was gradually raised to room temperature over a period of 2.5 hrs. After three washes of 100% methanol and three of 100% acetone the specimen

were infiltrated and embedded as described below.

c) <u>Air Drying Specimen Preparation</u>

Excised molars were allowed to air dry for 3 weeks. The molars were then washed in acetone and embedded in Spurr resin as described below.

3) <u>Embeddment and Microtomy</u>

After dehydration in methanol and acetone the specimens prepared for TEM were infiltated with Spurr resin in acetone (1:3,1:1,3:1,100%). Infiltration times were 4-6 hrs for each of the first 3 steps and overnight for 100% resin. The next day the specimen in 100% resin were placed for 15-30 min in a vacuum desiccator to remove air and then placed in an oven at 60°C for 8-12 hrs. Thick sections (0.5-1 μm) were cut with a diamond knife on a Porter Blum ultramicrotome, place on glass slides and stained with 1% aqueous toluidine blue to establish orientation. Thin sections (silver, 50-60 nm) were then cut and picked up on formvar-coated copper grids and were either stained (as described below) or left unstained. Cryoprepared specimens were embedded and oriented as described above; however, ultrathin sections, (transparent, <50 nm) needed for apatite analysis with selected-area dark field imaging, were cut with a diamond knife and were picked up on uncoated copper grids. Five to ten serial sections (grey/silver, 60-90 nm) were cut in different blocks and were observed unstained on formvar coated grids.

4) <u>Staining</u>

The mineralized components of the tooth have inherent electron density that can be visualized unstained by the electron microscope. The nonmineralized components and cells; however, are not sufficiently dense to provide ultrastructural definition, so heavy metal stains are used to enhance contrast. Conventionally prepared specimens on copper grids were suspended in 1% uranyl acetate in double distilled water (adjusted to pH 4.2) for 15 minutes, washed in double distilled water for 30-60 seconds, stained in Reynold's Lead for 10 minutes.

Specimens on formvar coated copper grids were demineralized with ethylenediaminetetra-acetic acid (EDTA) (Warshawsky and Moore 1967), washed, then stained with 1% phosphotungstic acid in double distilled water (pH 3.2) for 25 minutes, washed, then stained with 1% uranyl acetate in double distilled water (pH 4.2) for 10 minutes. These were then washed in distilled water before being air dried and viewed with the TEM. Some conventionally prepared tissue were not demineralized with EDTA, but were stained with phosphotungstic acid and uranyl acetate.

5) <u>Imaging Techniques</u>

Bright field (BF) TEM was used to image both stained and unstained specimens. In bright field EM images are formed by the unscattered electrons which pass through the specimen and are collected by the underlying objective aperture. In contrast, in a SADF image only the diffracted electrons are collected by the objective aperture. With crystalline materials, like apatite,

specific diffraction patterns are formed when electrons are transmitted through the specimen. For apatite these diffracted electrons give maxima (d-spacings) which can be collected by the objective aperture and in turn imaged. In this study electron reflections representing the ab- [102, 210, 211, 112, 300, 202, and 301 d-spacings], c- [002 d-spacing] and combined abc- axes of apatite were imaged by SADF. The ab- and c- axial reflections were selected using a 4 µm objective aperture, while the combined abc- axes were imaged using a 10 µm aperture.

Both the Philips 300 and 301 TEMs were used in bright field at accelerating voltages of 60 and 80 kV for stained specimens. The Philips 300 TEM was also used at 80 kV for bright and dark field images of unstained specimens.

RESULTS

Longitudinal planes of sections through the tooth at this early stage of development reveal monolayers of odontoblasts and ameloblasts secreting their respective extracellular matrices dentin and enamel (Fig. 1). This LM image of a 0.5 um thick plastic section provides an overview of odontogenesis and serves to focus attention on the DEJ which is the site of possible interaction between the organic components and apatite crystals of these two matrices. The odontoblasts and ameloblasts are separated by extracellular matrices of dentin and enamel. The enclosed areas (b,c,d,e) of this image correspond to representative areas observed at the EM level as shown in Figures 1b-e respectively. Figure 1b shows the pre-dentin matrix separated from an ameloblast by a BM, this matrix contains collagen fibrils, ground substance, matrix vesicles, and odontoblast processes. Collagen fibrils appear to narrow at their terminal portions which associate with the BM. Matrix vesicles have different staining properties and shapes depending upon their contents and they are usually found in close proximity to the BM (Slavkin et al., 1972). The ameloblasts which rest on the BM have ribosomes and vesicles in their apical cytoplasm. Figure 1c demonstrates the changes that occur in this region as predentin mineralizes: the BM has been broken down and remnants of it can be observed between outfoldings of the ameloblast apical membrane, and mineral deposition spreads from isolated loci within pre-dentin along collagen fibrils. Unfortunately due to routine staining procedures which result in the depletion of all detectable calcium phosphate and apatite; the previously

mineralized matrix can only be observed due to the structural modification of the matrix during mineralization which incorporates heavy metals during the staining procedure (Arsenault and Hunziker, 1988). In this micrograph, mineralization advances toward the apical membrane of ameloblasts along collagen fibrils which contribute to the uneven appearance of the surface of dentin at this stage. The more intense staining of the dentin surface appears to be analagous to the lamina limitans demonstrated in stain deposits of bone and cartilage by Scherft (1972). He has suggested that this elecron-dense layer that borders mineralized regions is formed as a result of accretion of organic material between and against apatite at the mineral surface, or by a process of adsorption.

The apical outfoldings of the ameloblast appear extended toward the pre-dentin and are in close proximity to collagen fibrils and an odontoblast process. The close proximity of an odontoblast process to an ameloblast gives evidence for a possible mesenchymal/epithelial cell-to-cell communication triggering the secretion of the enamel matrix (Slavkin and Bingas, 1976; Katchburian and Burgess, 1977). In the ameloblast, elecron-dense secetory product is stored in cytoplasmic vesicles near the apical cell membrane until mineralization reaches the edge of dentin. After mineralization reaches the surface of dentin the enamel matrix is exocytosed onto dentin (Fig. 1d). The initial enamel matrix has been described as stippled material because of its particulate nature (Reith, 1968). Enamel crystals are electron-dense needle-like structures which extend from the surface of dentin through the stippled enamel matrix toward ameloblasts. Initially these crystals are indistinguishable from

FIGURE 1 - <u>OVERVIEW OF THE FORMATION OF THE DENTINO-ENAMEL JUNCTION</u>

(a) A 0.5 µm thick section stained with toluidine blue shows five layers of cells. The stellate reticulum (SR), the stratum intermedium (SI), and the ameloblast layer (A) make up the supportive and secretory cells of enamel. The dental pulp cells (DP), and the odontoblast layer (O) are the supportive and secretory cells of dentin. Enclosed areas of interest are shown at higher magnification below. Bar = 20 µm; Mag = 800x.

(b) A portion of an ameloblast (A) is separated from the predentin matrix (PD) by a thin basal lamina (BL). Within the predentin matrix, and close to the basal lamina are matrix vesicles (MV), a cell process of an odontoblast (OP), and numerous randomly arranged collagen fibrils.

(c) During dentin (D) formation, mineralization occurs between and along collagen fibrils (small arrowheads). Here mineralization appears to be progressing toward the ameloblast (A) which has penetrated the basal lamina. An odontoblast process can be observed in close proximity to the apical membrane of an ameloblast. Within ameloblasts enamel products are stored in cytoplasmic vesicles (large arrowheads).

(d) The enamel matrix is released as a stippled material (SM) by the ameloblast (A) onto the surface of dentin (D), forming a layer

of enamel (E). The ameloblast is also involved in resorption, indicated by the presence of a pinocytotic-like vesicle (arrowhead).

(e) As more enamel matrix is released by the ameloblast the enamel layer (E) thickens. Bar = 500 nm; Mag = 36,000x. [b-e were conventionally prepared and stained with uranyl acetate and lead citrate (UA/LC)]
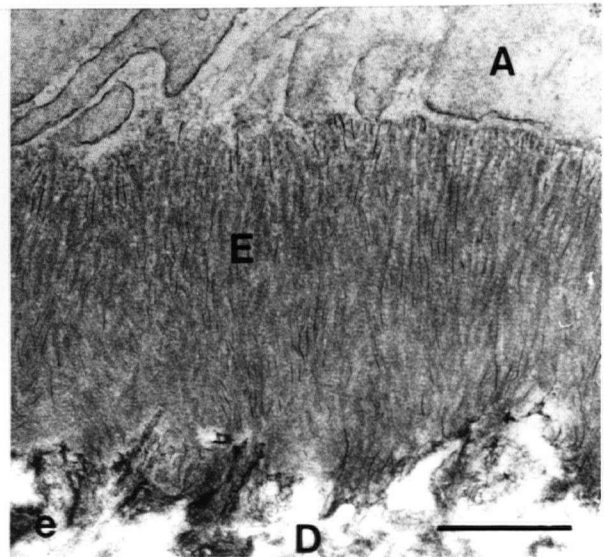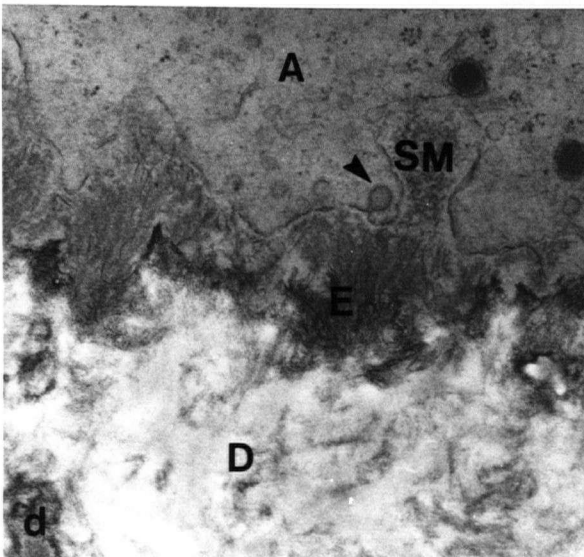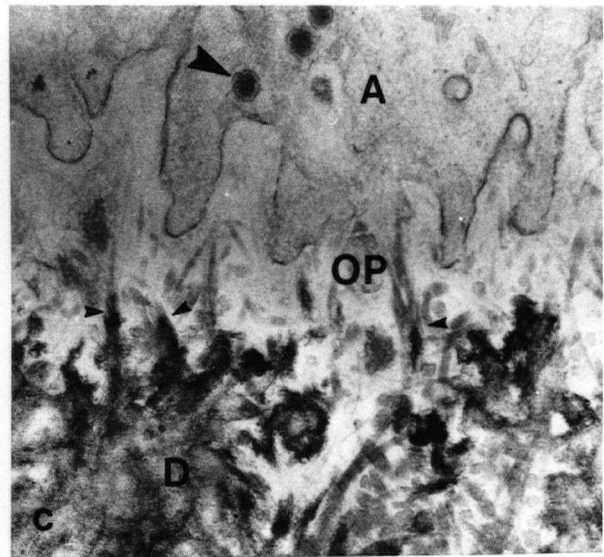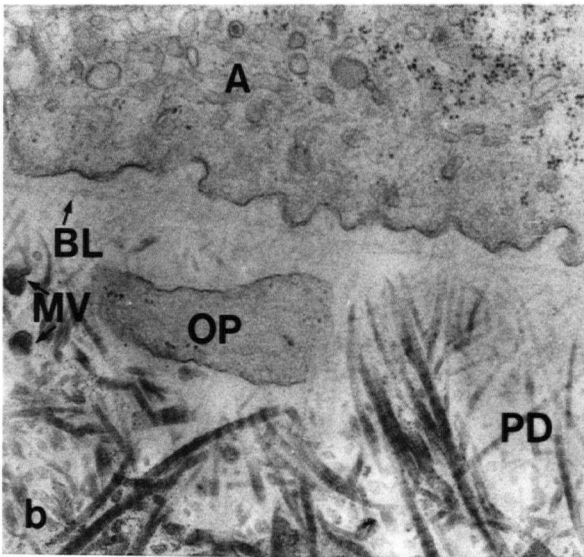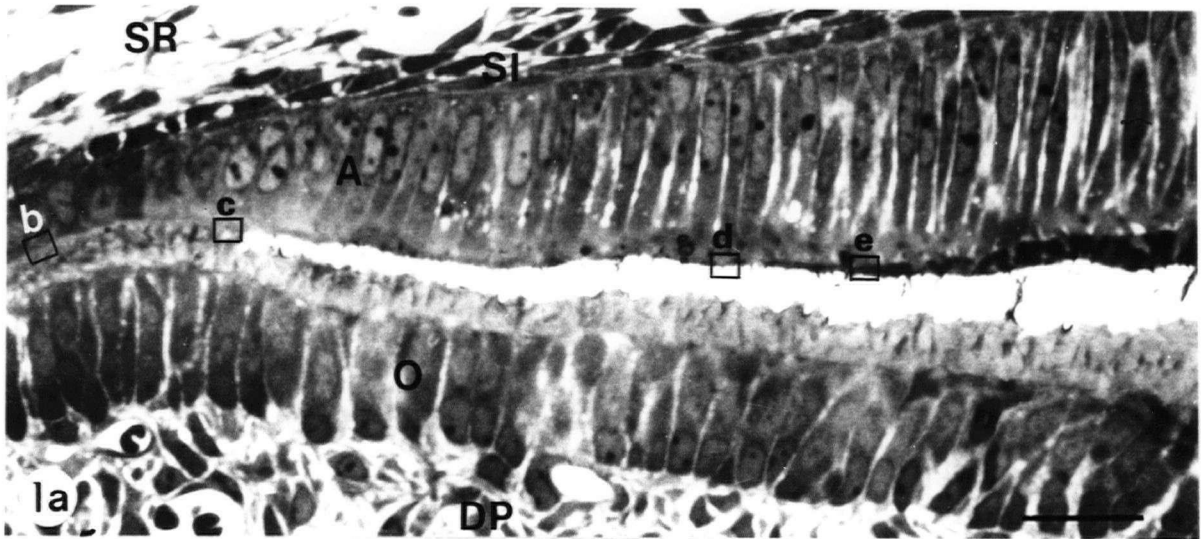
dentin crystals, except that they are observed within the enamel matrix. Further along the DEJ more matrix material has been released by the ameloblast and so the enamel layer is thicker; here the enamel crystals appear to be in close proximity to the apical cell membrane (Fig. 1e).

Electron densities resembling apatite crystals are first observed within matrix vesicles in pre-dentin (Katchburian, 1973). Figure 2b shows a matrix vesicle that is contained within the thickness of a section (60-90 nm thick) as this is the second section in a series of three. (Figs. 2a-c). These micrographs also demonsrate the extensive reticular fibrils associated with the BM and with the randomly arranged collagen fibers of pre-dentin. An unstained section of pre-dentin shows the radial arrangement of mineral deposition in three mineralizing loci (Fig. 2d). The smallest locus may be a matrix vesicle, but without staining to see a limiting membrane it is difficult to identify it from a cross-section of a mineralized collagen fibril. The electron-dense needle-like structures are dentin crystals and the electron-density surrounding these crystals may be due to an increased concentration of calcium and phosphate ions. Mineralization is initiated within matrix vesicles and then radiates through the afibrillar regions to collagen fibrils (Katchburian, 1973). Sometimes if the vesicle is very close to a collagen fibril, mineralization will follow the collagen fibril before radiating into the afibrillar region (Fig. 2e). This preferential mineralization of collagen fibrils, where mineralization proceeds along collagen fibrils more rapidly than through afibrillar matrix regions, has been reported by many authors in dentin and other collagenous tissue mineralization

(Arsenault, 1988; Glimcher, 1979; Höhling et al., 1976; Katchburian, 1973). This phenomena is not only observed in the formation of mantle dentin (first formed), but also is seen in the circumpupal dentin (subsequently formed) at the pre-dentin dentin border (Figs. 2f and g). A collection of collagen fibrils at the pre-dentin/dentin junction demonstrates the changes that occur upon mineralization (Fig. 2g). In the dentin, these fibrils appear larger and have an axial banding pattern which is more diffuse than those in pre-dentin. Figure 2g is a lower magnification of a region similar to Fig. 2f showing the pre-dentin/dentin border without staining. The dense area in the centre of the dentin results from the mineralizaton of the closely packed collagen fibrils in a von Korff fibre oriented perpendicular to the pre-dentin/dentin border. The von Korff fibres appear to be responsible for extensions of mineralization into pre-dentin (Bernard, 1972), suggesting that mineralization grows more rapidly along the organized collagenous matrix than through the less structured afibrillar matrix.

Figures 3a and b are micrographs of the pre-dentin/ameloblast junction at a stage just prior to the secretion of enamel matrix (Fig. 1c). The BM has been removed and projections from ameloblasts extend into pre-dentin, between these projections javelin pointed collagen fibrils can be observed. Pinocytotic-like vesicles in the ameloblast near its apical membrane suggest that it is involved in resorption of the BM as postulated by Reith (1967). At a later developmental stage, after a prolonged demineralization and staining, collagen fibrils are discernable from the rest of the mineralized matrix (Fig. 3c). Unlike the collagen fibrils of Figs. 3a and b, these fibrils have a larger

FIGURE 2 - <u>INITIATION AND SUBSEQUENT MINERALIZATION OF DENTIN</u>

(a-c) A series of micrographs of serial sections, showing a matrix vesicle (MV) to be within the thickness of the second section. An ameloblast (A) rests on a basal lamina (BL). Collagen fibrils (small arrowheads) in the predentin are randomly arranged observed here cut in transverse and cross section (a) to be distinct from the odontoblast process (OP).   Bar = 200   nm; Mag = 52,000x. [conventionally prepared and stained with UA/LC]

(d) An unstained electron micrograph of conventionally prepared predentin shows three progressive stages of mineralization. The arrowhead points to an electron-dense region surrounding a single apatite crystal, probably a matrix vesicle. The other two electron-dense areas contain more mineral, which appears to radiate from a central point. Two ameloblast cellular projections can be observed above the predentin. Bar = 200 nm; Mag = 78,000x.

(e) Mineralization in dentin progresses from matrix vesicles (arrowheads) to collagen fibrils, through the afibrillar matrix, then along the randomly arranged collagen fibrils. Bar = 400 nm; Mag = 47,000x. [conventionally prepared and stained with UA/LC]

(f) Organized collagen fibrils of a von Korff fibre demonstrate the predentin (PD)/dentin (D) border. The distinct periodic banding of the pre-dentin portion of these collagen fibrils

appear to be reduced in the dentin portion. Bar = 150 nm; Mag = 77,000x. [conventionally prepared and stained with Phosphotungstic Acid (PTA)/UA]

(g) A lower magnification of an unstained section in a similar area to Figure 2f. A von Korff fibre (VK) is more densely mineralized than the surrounding dentin (D). Bar = 200 nm; Mag = 31,000x. [conventioally prepared and not stained]
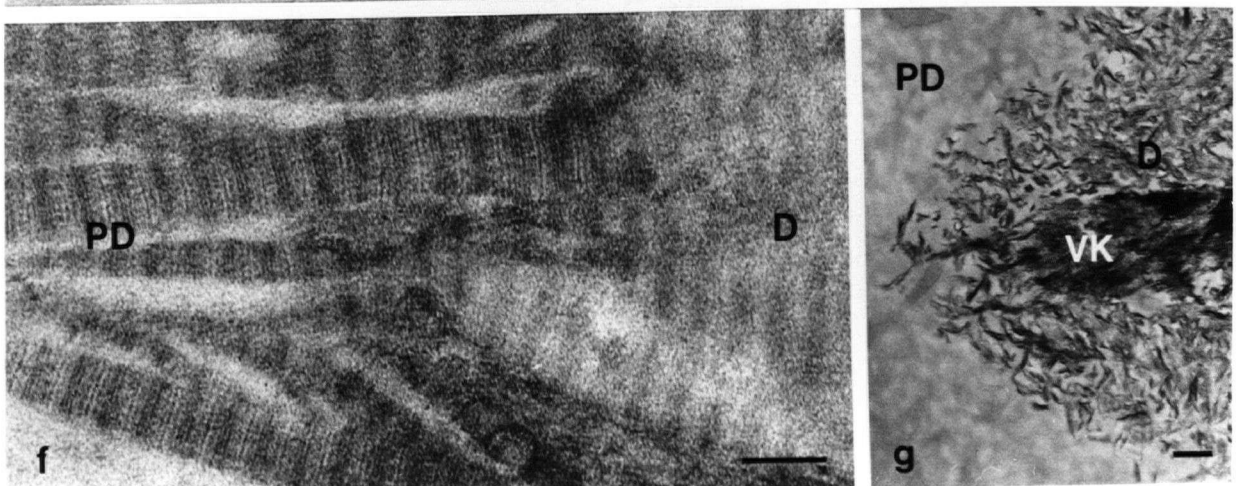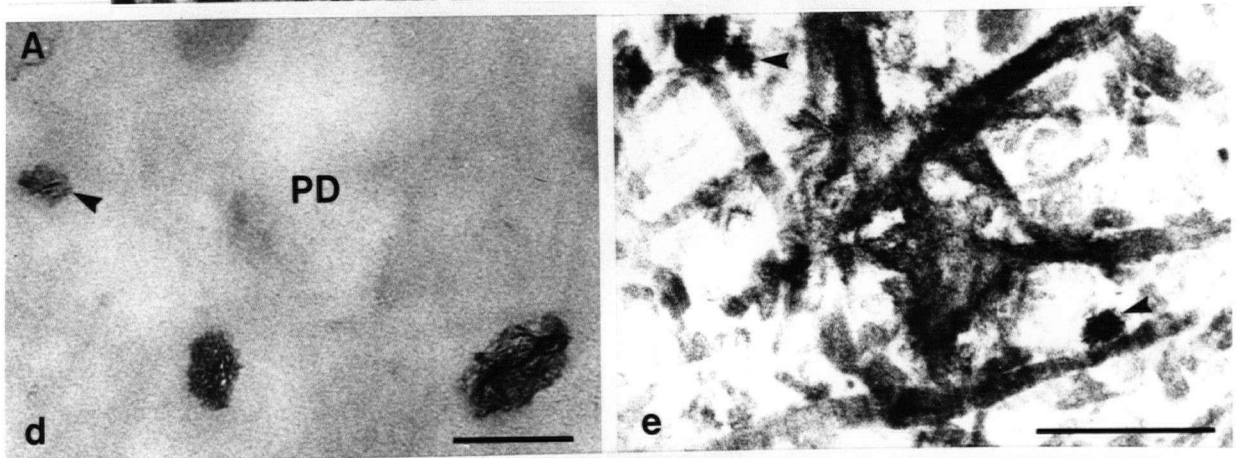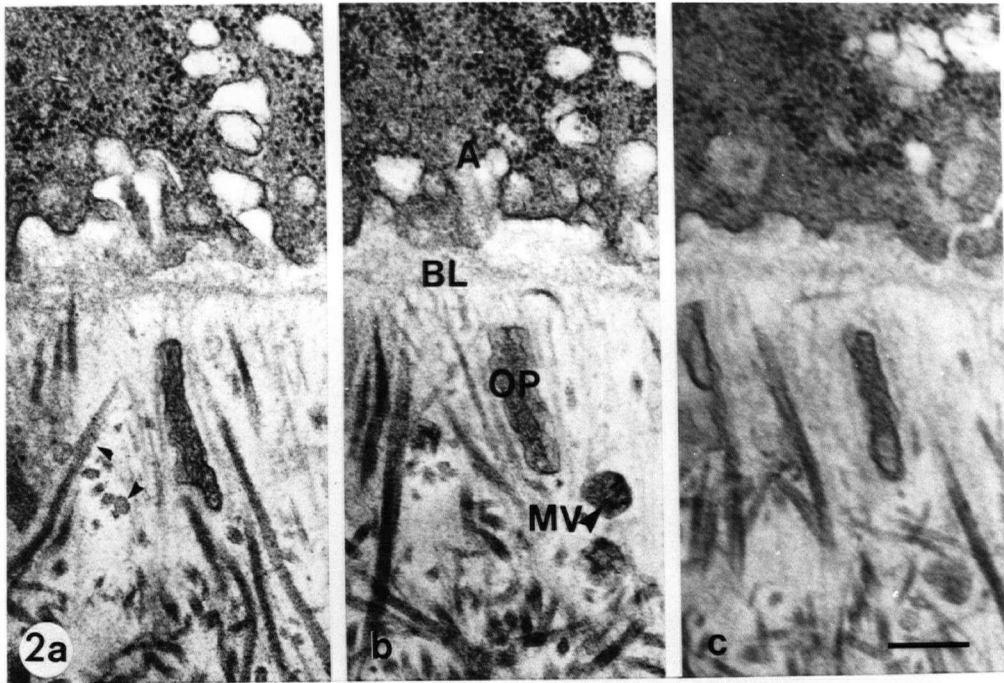
diameter (1 to 2 times in the main portion and 5 to 12 times for the terminal portion), and a reduced axial banding in their terminal portion which appears to be frayed rather than pointed. Thin electron-lucent lines in the dense enamel matrix are spaces occupied by enamel crystals prior to demineralization, they are found to be associated with the terminal, frayed portion of dentin collagen fibrils.

Figure 4 contains a galley of electron micrographs that show the sequential stages of transition along the developmental gradient at the DEJ. At a developmental stage between those shown in Figs. 1b and c, remnants of the BM are still present between apical projections of ameloblasts. In the pre-dentin matrix a locus of mineralization appears to have been initiated from a single point, probably a matrix vesicle (Fig. 4a). The electron density of collagen fibrils associated with this locus demonstrate the radial pattern of mineralization. As mineralization progresses this locus of mineralization will become larger and coalesce with others like it to form a continuous layer of mantle dentin seen in Fig. 4b. Just before the ameloblast enters the secretory phase the surface of dentin appears uneven due to collagen fibrils jutting out from the surface of mineralized dentin (Fig. 4b). When the ameloblast begins to release enamel matrix onto the dentin surface, the matrix appears to bathe newly mineralized collagen fibrils (Warshawsky 1985). Enamel crystals form on the surface of the dentin and grow into the stippled enamel matrix towards the ameloblast (Figs. 4c). Along the DEJ the distance between the ameloblast layer and the surface of the dentin is increased due to the subsequent matrix release and crystal growth

FIGURE 3 -   <u>CHANGES IN COLLAGEN AS THE DEJ FORMS</u>

(a,b) High magnification electron micrographs of the apical region of an ameloblast (A) show javelin pointed ends of collagen fibrils to be in close proximity to the ameloblast. (a) Pinocytotic-like vesicles (large white arrowhead) observed suggest some matrix resorption is taking place. (b) This alteration occurs as mineralization progresses from the dentin (D) towards the ameloblast along the fibrils.

(c) Prolonged demineralization and staining exposes similar collagen fibrils (asterisks) that are now bordered by enamel (E). The ends of these fibrils in dentin (D) have a reduced axial banding, and an expanded fibrillar appearance. Lucent areas (small white arrowheads) in the enamel matrix were formerly occupied by enamel crystals. Bar = 200 nm; Mag = 99,000x. [conventionally prepared, demineralized with EDTA and stained with phosphotungstic acid (PTA)/UA]
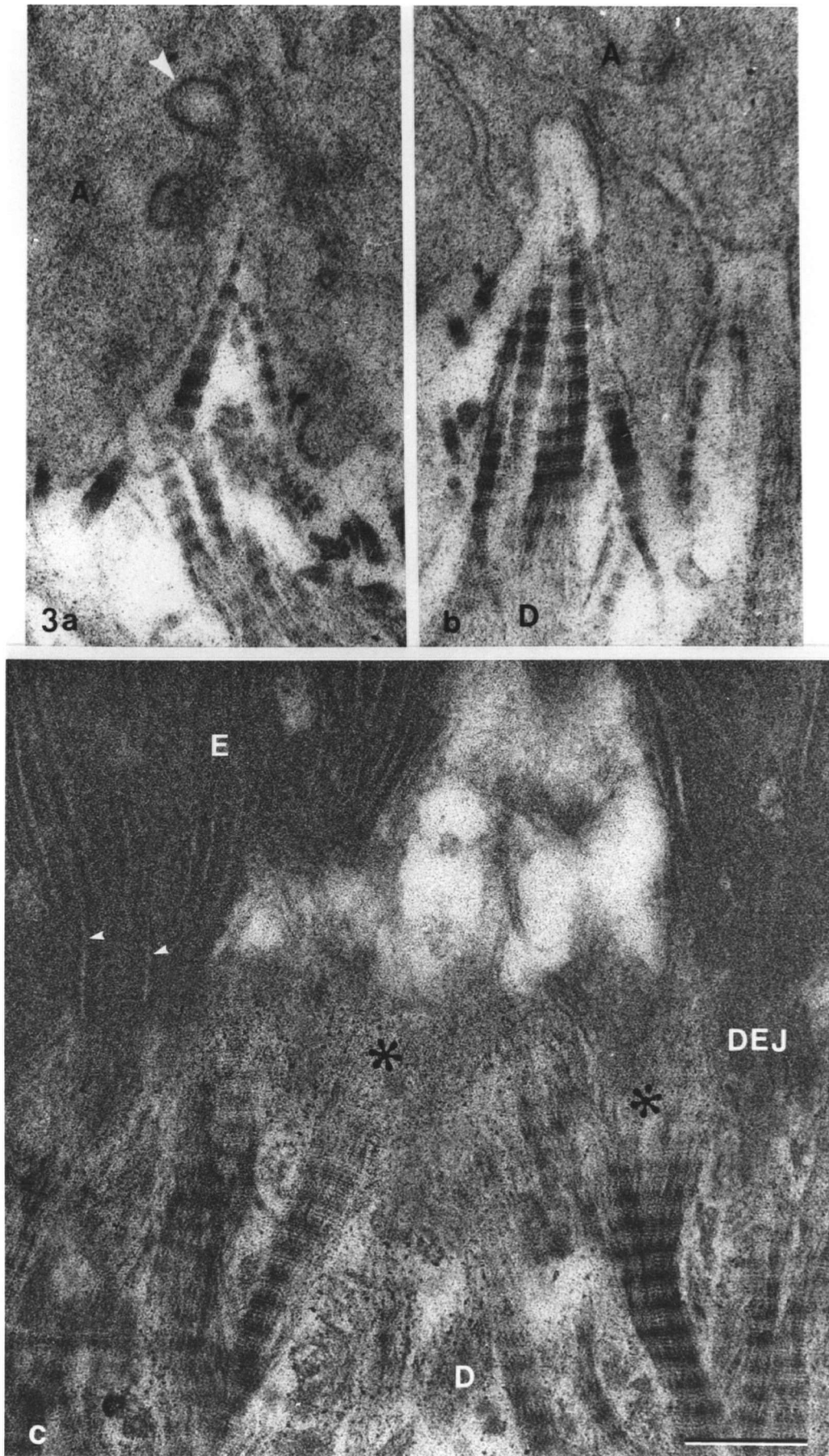
(Figs. 4d-f) (Osborn 1972). After a uniformly thick layer has been formed the ameloblast developes the Tomes' process, an apical projection which directs the orientation of enamel crystals into rod and interrod (Figs. 4e-f). The presence of a large number of electron-dense vesicles within a Tomes' process indicate the large amount of enamel matrix which is secreted during the formation of enamel.

Sections of the DEJ were demineralized and then stained so that all detectable mineral would be removed so that the underlining organic structure could be observed in both cryogenic (Fig. 5a) and conventional (Fig. 5b) preparations. In both preparations a 5 nm wide electron lucent space can be observed where the enamel crystal existed prior to demineralization. The electron-dense material surrounding the space is the enamel sheath (Fig. 5a), other authors have observed this structure and its relationship with enamel crystals (Jessen, 1968; Travis and Glimcher, 1964). The enamel sheath appears to be intimately associated with the surface of dentin (Fig.5a) and specifically with the frayed end of a collagen fibril (Fig. 5b). Individual sheaths extend from the collagen fibril to the edge of the enamel matrix just below the ameloblast cell membrane.

Figure 6c is an unstained bright field image of the DEJ, it is approximately the same stage of development as Figs. 1e, 4f, 5a, and b. Enamel and dentin apatite crystals are imaged here due to their electron density, the much less electron-dense organic matrix is not seen. Confirmation that these two groups of crystals are apatite comes from their electron diffraction patterns which have the typical apatite d-spacings (Figs. 6a and b). The diffraction patterns of both

FIGURE 4 - SEQUENTIAL STEPS OF ENAMEL MINERALIZATION

(a) Before the enamel matrix is released a locus of mineralization can be observed in the predentin matrix (large arrow). Mineralization appears to spread epitaxially along collagen fibrils. Processes of ameloblasts (A) extend into the predentin matrix.

(b) As mineralization reaches the ends of collagen fibrils at the surface of dentin the enamel matrix is released (small arrow).

(c) The electron-dense enamel matrix covers the surface of the dentin, and soon after it is released. Enamel crystals, which appear as thin electron-dense lines, are seen to have extended from the dentin toward ameloblasts. The crystals appear only to grow into regions where there is enamel matrix.

(d-f) Further along the DEJ more enamel matrix is secreted and the ameloblasts appear to move away from the surface of dentin. The enamel crystals grow to be in close proximity to the surface of the ameloblasts (curved arrows). Bar = 500 nm; Mag = 35,000x. [conventionally prepared and stained with UA/LC]
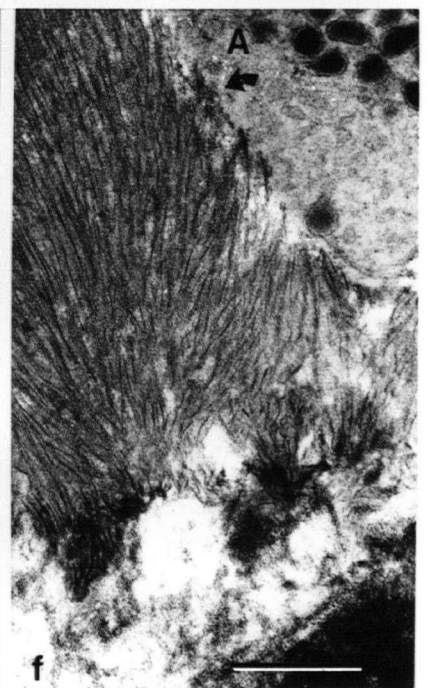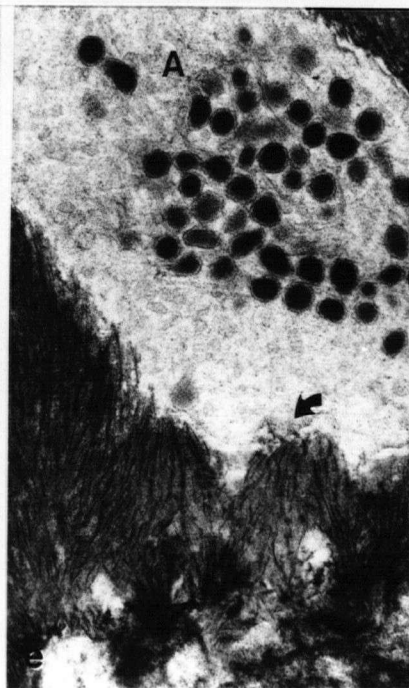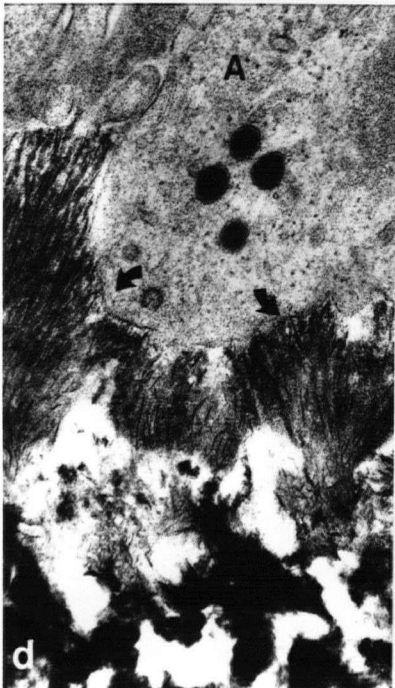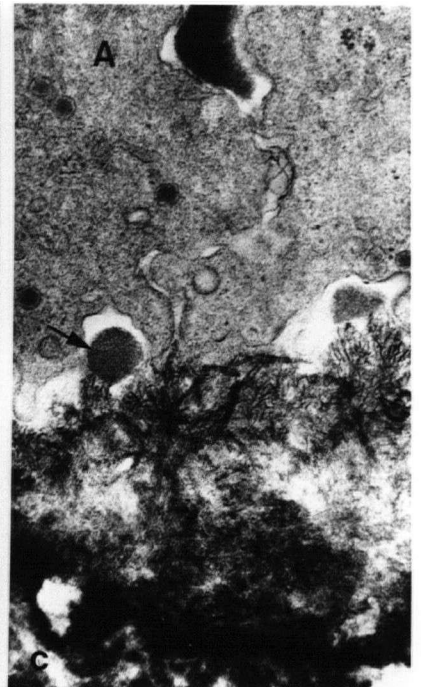
FIGURE 5 - <u>THE ENAMEL SHEATH</u>

(a) A cryogenically prepared, demineralized/stained section of the DEJ shows enamel sheaths within enamel (E). These sheaths are associated with the surface of dentin (D) (large white arrowhead). The diameter of the sheath is 5nm at this stage of development (small black arrowheads).

(b) A similar section conventionally prepared shows that the sheath in enamel (E) is associated with the frayed end of a collagen fibril (large white arrowhead) within dentin (D). The diameter of these sheaths is 5nm. There appears to be some space between the plasma membrane of the ameloblast and the end of the sheath. Bar = 100 nm; Mag = 132,000x. [both demineralized with EDTA and stained with PTA/UA]

5a   b
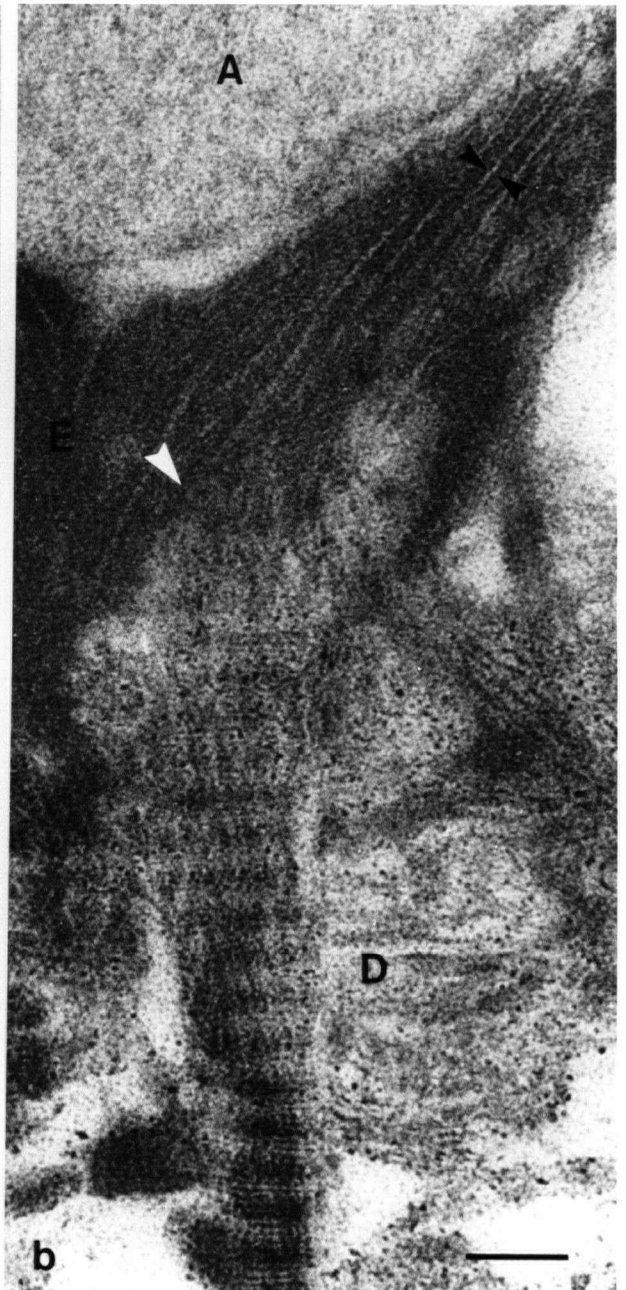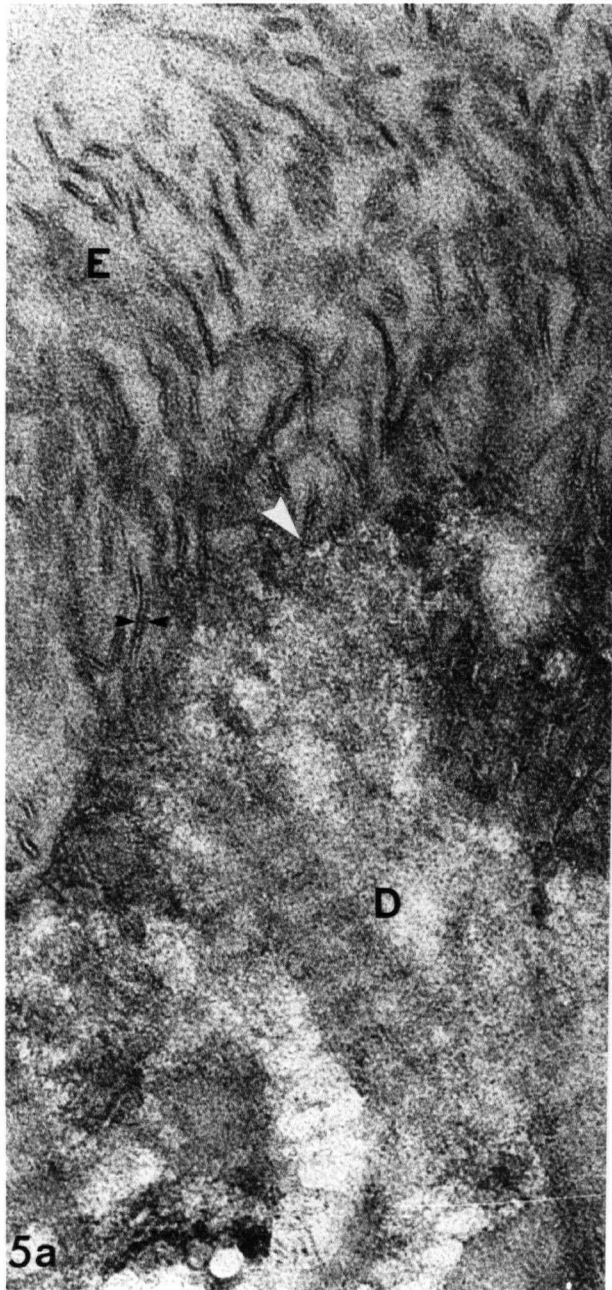
enamel (Fig. 6a) and dentin (Fig. 6b) have maxima of the same spacing indicating that their unit cell dimensions are the same, however the pattern for enamel is less intense than that of the dentin. This difference is due to the sparse distribution of enamel crystals which are 5 nm in width and vary in length at this stage as compared to the smaller more densly packed dentin crystals. An intimate spatial relationship between enamel and dentin crystals can be observed in Fig. 6c. This relationship is not limited to the surface of dentin, but can also be observed in the dentinal furrows between collagen fibrils. Mineralized collagen fibrils appear blunt-ended as they jut out from the surface of the dentin. A selected-area dark field (SADF) image of the same area (Fig. 6d) demonstrates this relationship as well. Enamel crystals can be distinguished from dentin crystals at the DEJ due to their larger size and positioning. Crystal continuity between dentin and enamel is more clearly demonstrated with SADF imaging because the image shows only the apatite. Figure 6d demonstrates at the different locations along the DEJ larger enamel crystals are intimately associated with the smaller dentin crytals.

Selected-area dark field can also be used to demonstrate a crystal organization in dentin that is not apparent in bright field imaging due to its heavy mineralization. Figures 7 a-d give an overview of the mineral component of dentin with different modes of imaging, at a developmentally more mature DEJ than in Fig. 6. Figure 7a is an unstained bright field image of the DEJ, crystals within the dentin appear to be randomly arranged, except along heavily mineralized collagen fibrils. The SADF image of this area formed by the ab+c-axial reflections does not add any information to the bright

FIGURE 6 - <u>CRYSTAL CONTINUITY- NEWLY FORMED DENTINO-ENAMEL JUNCTION</u>

(a-b) Diffraction patterns of newly formed apatite in (a) enamel and (b) dentin demonstrate the maxima (reflected electrons) of the c axis (1) and a combined ab+c axes (2). The maxima of initial enamel appear more faint than those of dentin at this stage of development.

(c-d) Companion bright field (c) and selected-area dark field (d) images of the DEJ at a stage similar to Figures 1e and 4f. (c) Only the high mass densities of apatite crystals in both enamel and dentin are visualized. A mineralized collagen fibril (C) projects into the enamel matrix. (d) A combined axial image shows a close association between dentin and enamel apatite crystals. Arrows (black and white) delineate this close spatial relationship between dentin and enamel crystals. Bar = 100 nm; Mag = 138,000x. [cryogenically prepared; unstained]. (images c and d courtesy of Dr. A.L. Arsenault)
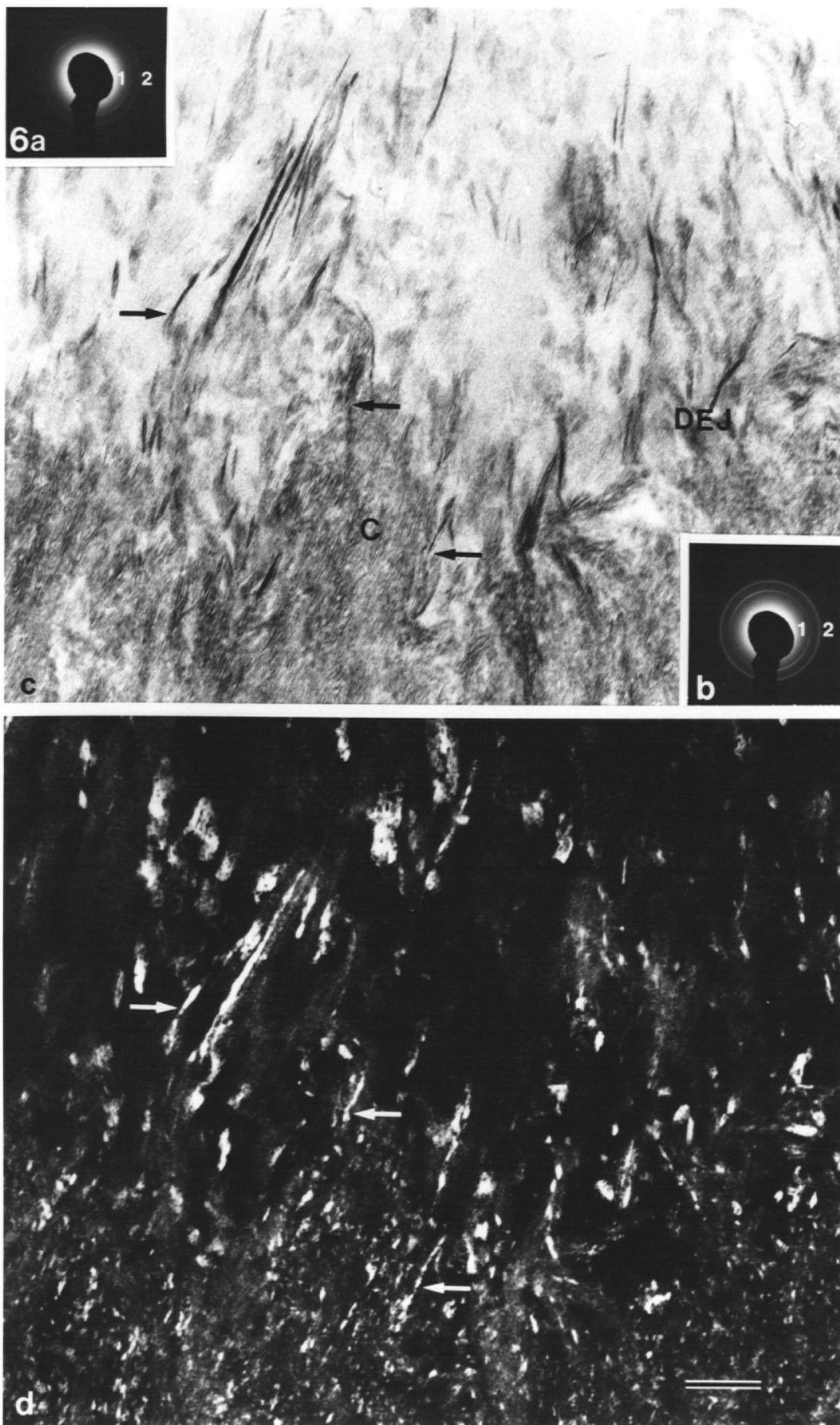
FIGURE 7 - <u>CRYSTAL ORGANIZATION IN DENTIN</u>

(a) A bright field image of the DEJ at a later stage of development demonstrates mineralized collagen fibrils (C) within dentin (D) and larger apatite crystals within the enamel (E).

(b) This same area is shown in an a,b + c-axial dark field image which demonstrates the distribution of apatite crytals within dentin and enamel.

(c-d) These dark field images selectively discriminate a,b-axes and c-axis. The numbers (1-3) correspond to the different orientations of crystals within dentin. The crystal organization in the c-axis dark field image (d) corresponds to the mineralized collagen fibrils in the bright field image (a). Bar = 200 nm; Mag = 47,500x. [cryogenically prepared; unstained]
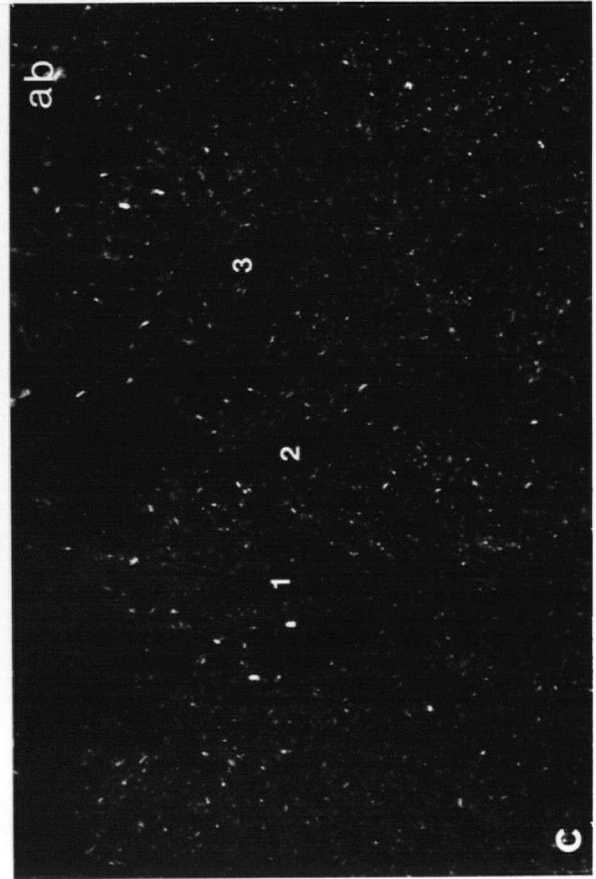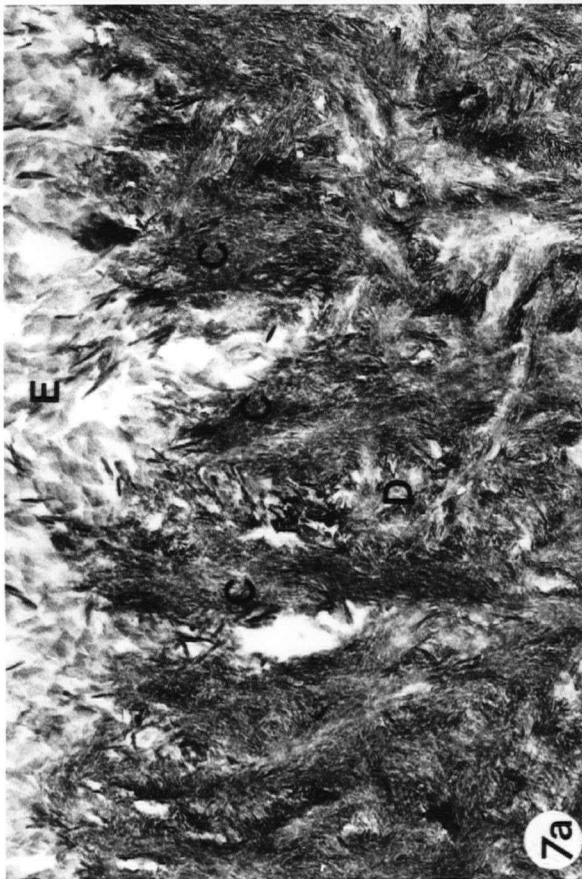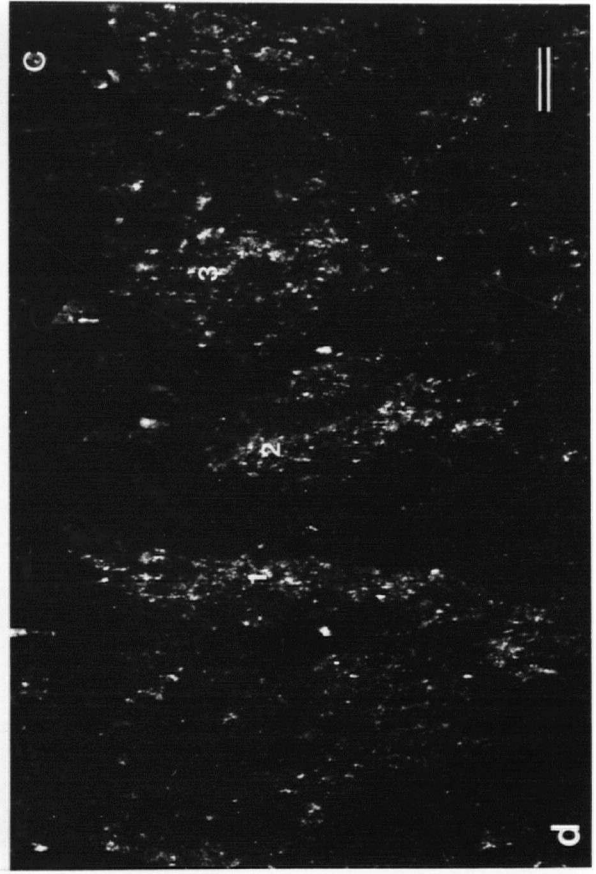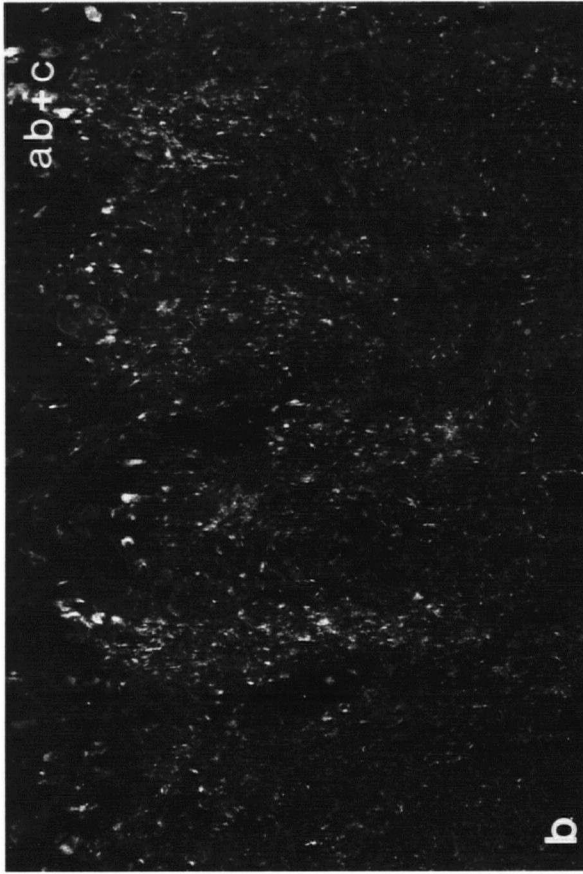
field image (Fig. 7b). The dark field ab-axes image shows a mostly random distribution of crystals and some spaces where there are no crystals, these correspond to areas of c-axially oriented crystals in Fig. 7d. Figure 7d is a dark field image made up of reflected electrons from the c-axis lattice plane of apatite crystals which are lying perpendicular to transmitted beam. Dentin apatite crystals are seen to be oriented along some collagen fibrils, but not others. Curving of the fibrils out of the plane of the image, overlaying of other fibrils or changes of orientation of crystals within the collagen could have these results. It should be noted that SADF images only a portion of the total apatite crystal distribution within dentin and enamel because the objective aperture which is used to select apatite lattice reflections from the diffraction pattern is of limited diameter, therefore it selects only a small portion of the maxima of reflections that result from a random organization of crystals.

Maturation is observed in later developmental stages by the increased width of enamel crystals (Fig. 8a). Maturation is a process whereby the amount organic matrix is reduced while the inorganic material is increased. These wider enamel crystals (25 nm wide) can easily be distinguished from the smaller dentin crystals (5 nm wide). As in the less mature enamel (Fig.6a), these enamel crystals are in an intimate spatial relationship with dentin crystals, not only in the heavily mineralized collagen fibrils, but also in between these fibrils within dentin furrows. SADF, using reflections from the ab+c-axes, of the DEJ at the same stage also demonstrate spatial intimacy between enamel and dentin crystals (Fig.8b-d).

FIGURE 8 - <u>CRYSTAL CONTINUITY- MORE MATURE DENTINO-ENAMEL JUNCTION</u>

(a) As the DEJ matures the crystals within enamel (E) increase in size. They are observed in this bright feild image to be associated with the dentin (D) not only its surface (large arrow), but also deep within dentinal furrows between mineralized collagen fibrils (C) (small arrows).

(b-d) A combined axial image demonstrates the same crystal continuity between apatite crystals of dentin and enamel as observed at the newly formed DEJ (Fig. 6d). The close spatial relationship between dentin and enamel crystals (white arrowheads) is clearly evident because of the greater difference in crystal size at this stage of development. Bar = 100 nm; Mag = 93,000. [air dried; unstained]
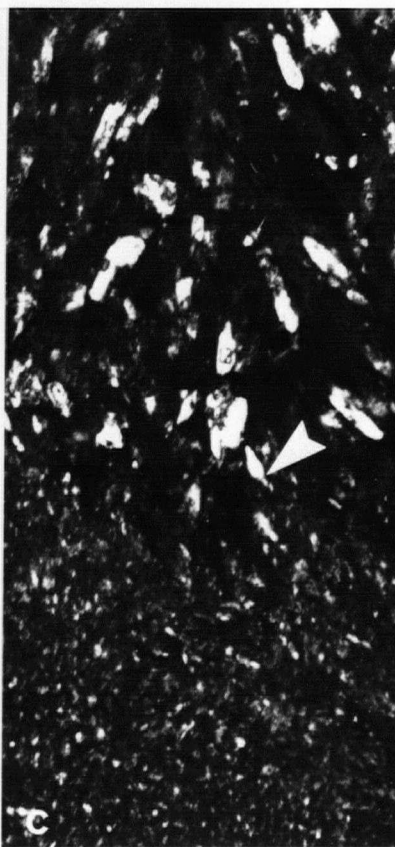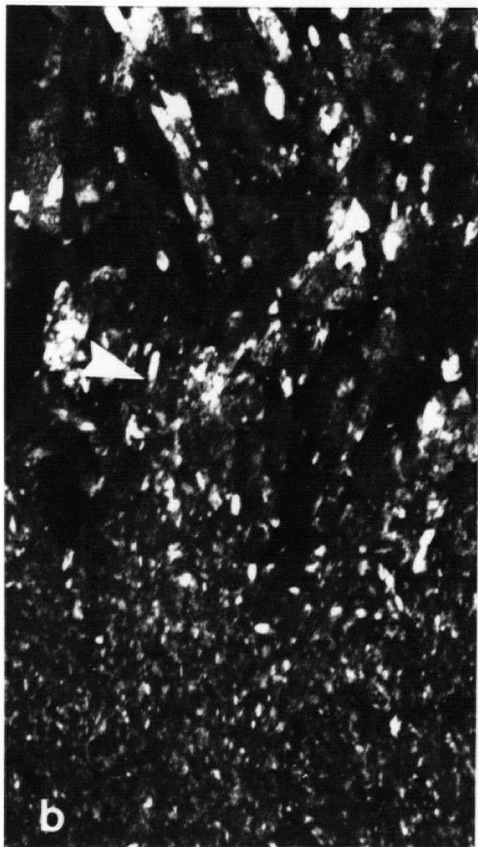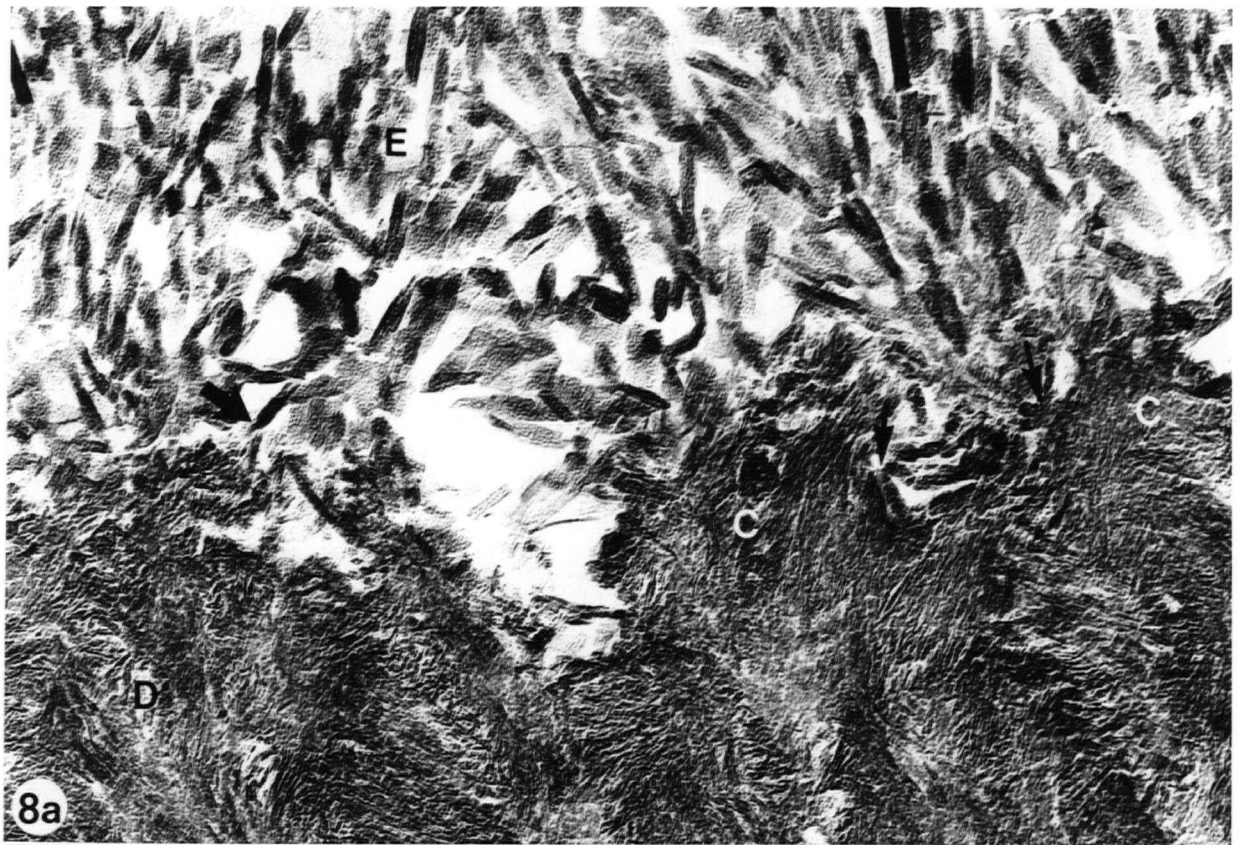
FIGURE 9 - <u>SCHEMATIC REPRESENTATION OF DENTINO-ENAMEL JUNCTION</u>

<u>FORMATION</u>

This is a schematic representation of the major events leading

to and including the initiation of enamel mineralization. (a) Prior

to mineralization of dentin ameloblasts (A) which contain protein

secretory apparatus are associated with a basal lamina (BL). Within

the pre-dentin (PD) collagen fibrils (C) occur in a random

arrangement, between them are matrix vesicles (MV) and odontoblast

processes (OP). (b) Mineralization of dentin (D) is initiated after

the breakdown and partial removal of the basal lamina. Initiation of

dentin mineralization arises within matrix vesicles and the epitaxial

spread of dentin apatite crystals follows collagen fibrils as it

proceeds toward ameloblasts. Once mineralization reaches the edge of

dentin, ameloblasts begin to exocytose the enamel matrix which has

been stored in secretory vesicles (SV). Pinocytotic vesicles (PV)

that appear at the apical membrane of ameloblasts are thought to be

involved in the breakdown and resorption of the basal lamina. They

may also have a role in resorbing the terminal portions of pre-dentin

collagen fibrils associated with the basal lamina. (c) Enamel apatite

crystals (EC) grow from the edge of dentin through the enamel matrix

(EM), toward ameloblasts. (d) The area bounded by a box in (c) is

magnified to show the interpreted relationship between dentin and

enamel. This plate is a working model of the spatial relationship

between both the organic and the inorganic components of dentin and

enamel. In this figure the collagen molecules of the frayed collagen

fibril appears to be presenting dentin apatite crystals (DC) to the enamel matrix. Some of these dentin crystals become enamel crystals as they form within enamel sheath (red). The intimate spatial relationship between dentin and enamel crystals is delineated by a large arrowhead.
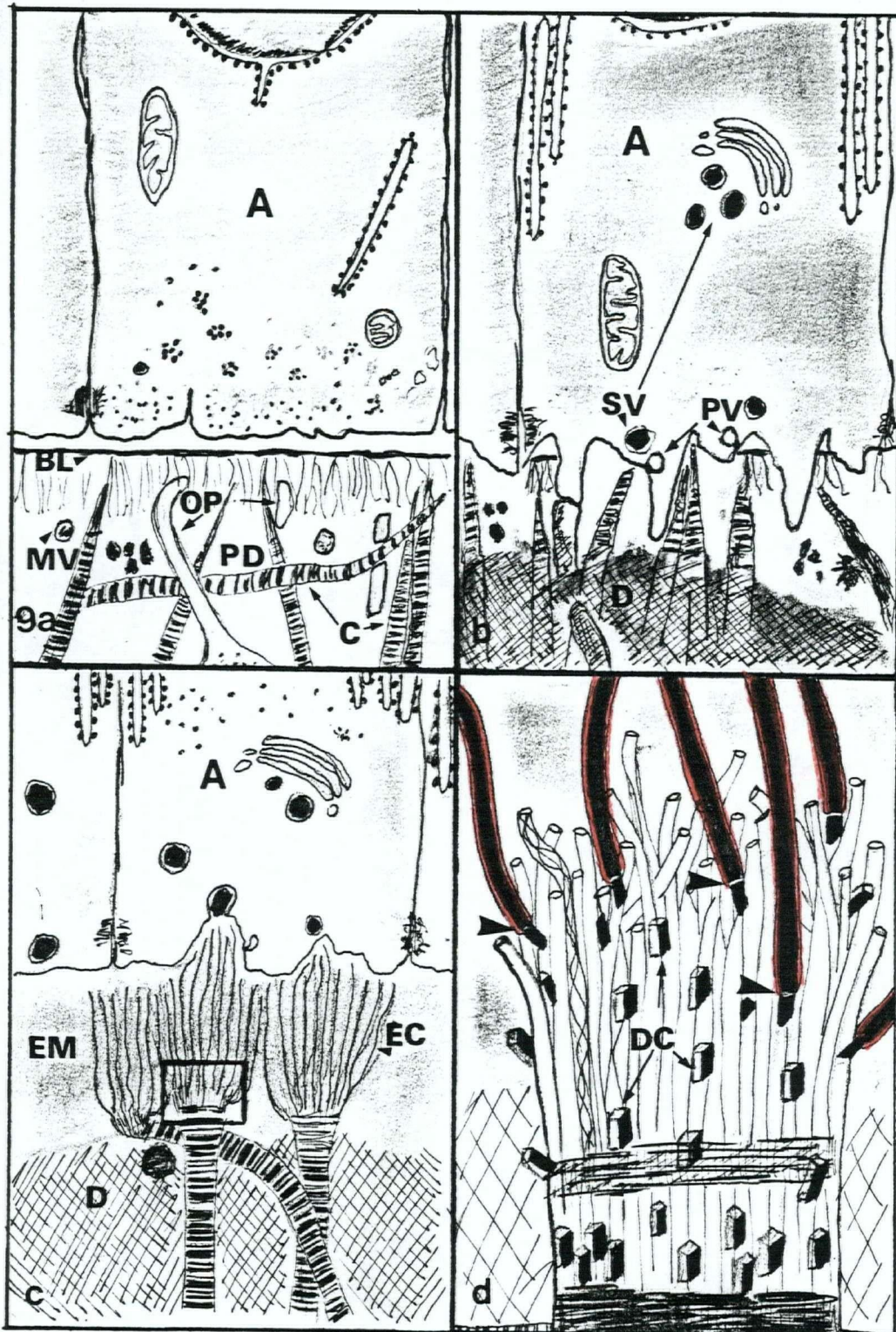
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DISCUSSION

The purpose of this thesis which examines the formation of the dentino-enamel junction (DEJ) was to find out how biological apatite crystals of enamel are initially formed. Information from other authors suggests that the initiation of enamel formation is different from both dentin and bone because the enamel matrix does not contain matrix vesicle or collagen fibrils, as well dentin is shown to form prior to enamel, but the initiating mechanism has not been proposed (Eisenmann and Glick, 1972; Reith, 1967; Ronnholm, 1962). Of all the authors who have presented an electron microscopic (EM) view of the development of the DEJ, only Bernard (1972) has suggested that enamel mineralization occurs as a secondary mineralization process which is reliant upon dentin mineralization based upon evidence of a close spatial and temporal relationship between crystals of dentin and .enamel. Extrapolation of his hypothesis leads to the suggestion that enamel crystals may be extensions of dentin crystals which grow into the enamel matrix. Evidence for this extended hypothesis which would be a direct crystal continuity between dentin and enamel crystals is examined in this thesis. In this thesis I will show evidence that this continuity exists and present a model to summarize the involvement of both dentin and enamel organic matrices in providing an environment conducive to forming crystal continuity (Fig. 9).

Before describing how the DEJ is formed the importance of the intimate spatial relationship between dentin and enamel must be established by presenting some functional possibilities from this interaction. Dentin may provide a structural foundation onto which

the enamel can form, a chemical bonding may occur between the two organic matrices while crystal continuity is established. The firm attachment of enamel to dentin is required from the tooth's function in mastication. Dentin may be to the enamel as matrix vesicles are to dentin, acting as a nucleating agent for enamel crystals; organic/inorganic components of dentin may alter the initially released enamel matrix so that it will mineralize. Alteration of the predentin matrix at the dentino-ameloblast junction (DAJ) prior to enamel matrix release may allow the direct exposure of dentin crystals to the enamel matrix promoting an epitaxial growth of enamel crystals. Enamel crystals which interdigitate with the uneven surface of dentin would also promote a strong attachement of enamel to dentin. These functional possibilities show a reliance of enamel on the normal formaton of dentin, this is evident in pathological conditions where dentinal dysphasias (dentinogenesis imperfecta, opalescent dentin, and shell teeth) affect enamel formation or its attachement to dentin (Rushton, 1954; Suzuki et al., 1977; Levin et al., 1983; Clergeau-Guerithault and Jasmin, 1985). A case study of a dental dysphasia, opalescent dentin, showed the DEJ to be a straight line rather than its regular scalloped appearance, the layer of enamel was hypomineralized and much thinner than in the normal condition it also lacked the rod/interrod arrangement of crystals (Susuki et al., 1977). These abnormalities of the enamel were in addition to the poorly formed dentin of this condition. In many of these dysphasias there are less severe variants where the enamel layer appears normal, this only occurs in conjunction with the normal formation of the mantle layer of dentin (Witkop, 1971).

The developmental sequences of dentin and enamel are presented in this thesis in the following order of events. (1) Matrix vesicles within pre-dentin that initiate mineralization contain crystal-like electron-dense material. These matrix vesicles are positioned between collagen fibrils that are randomly arranged; the basal lamina between pre-dentin and ameloblasts is intact (Figs. 1b and 2b). (2) The basal lamina is penetrated by ameloblast cell processes and there is an increase in the amount of apatite crystals in the area where the initial matrix vesicles were. Mineralizing regions in pre-dentin are described as loci (Hayashi, 1983; 1984; Figs. 1c and 4a). (3) As more crystals form, there is a spread of mineralization from initial loci in afibrillar regions to collagen fibrils, and then epitaxially along these making the loci larger (Figs. 2d, e and 4a). (4) Mineralization proceeds along collagen fibrils toward ameloblasts which begin to secrete an electron dense product which has been synthesized and stored in vesicles near the apical cell surface. The enamel matrix covers the surface of dentin (Fig. 4b). (5) Soon after the enamel matrix is released apatite crystals form on the surface of the dentin and begin to grow toward the ameloblast, only in areas where the enamel matrix is present (Figs. 1d and 4c). (6) As more matrix is released the enamel crystals grow to be in close proximity to the apical surface of the ameloblast (Figs. 4d-f) (Reith, 1967; Bernard, 1972; Eisenman and Glick, 1972; Katchburian, 1973, Slavkin and Bringas, 1976).

These events suggest that there is a progression of mineralization initiated in dentin and continued in enamel. Collagen may have a role in presenting mineral in dentin to enamel because

mineral associated with collagen fibrils is the first to come in contact with new released enamel matrix (Figs. 1c, 2e, and 4a-c), and the fibrils are known to organize apatite crystals with their c-axis parallel to the c-axis of the fibril (Fig. 7; Arsenault, 1988).

The active involvement of dentin collagen fibrils in the presentation of dentin apatite to the enamel matrix may be related to changes in the appearance of terminal portions of collagen fibrils during the formation of the DEJ. Before complete mineralization of predentin the terminal portions of collagen fibrils associated with the reticular lamina of the BM have been described as javelin-like in appearance (Figs. 1b, 2a-c, and 4a; Bernard, 1972). After the BM is removed these fibrils are closely associated with the apical surface of ameloblasts which extend into the predentin matrix (Fig. 3a, and b). After mineralization the terminal portions of dentin collagen fibrils dramatically change; removal of mineral from a longitudinal thin section of the DEJ show these fibrils to be frayed at the ends previously associated with the BM. It appears as if the intermolecular cross-links between collagen molecules break to give the unravelled appearance of the terminal portions of these collagen fibrils in the presence of the enamel matrix. These observed changes to these collagen fibrils may result from a change in their functional significance. Prior to mineralization, javelin-pointed collagen fibrils may have a role in anchoring the BM giving the odontoblasts, which produce this collagen, an indirect control over the orientation of ameloblasts which rest on the BM (Bernard, 1972). This anchoring of connective tissues to basement membranes produced by epithelial cells has been documented in other tissue systems in a recent study by

Inoue and Leblond (1988). After the BM has been removed the anchoring of ameloblasts by collagen fibrils appears to continue until the enamel matrix is secreted (Figs. 3a and b), but during this mineralization of the dentin matrix terminal portions of collagen fibrils become frayed in appearance (Figs. 3c and 5b). The role of these remodelled collagen fibrils may be in presenting apatite of dentin to the enamel matrix which is released upon them, but the frayed collagen fibrils may also contribute to the formation of a strong bond between the dentin and enamel at the DEJ.

A possible explanation for the changes occurring to the terminal portions of these collagen fibrils is that odontoblast derived collagenase, stored in matrix vesicles in pre-dentin, is released during mineralization of dentin and degrades the ends of the collagen fibrils giving them their frayed appearance. Evidence for this explanation is provided by a study which found collagenase activity associated with the contents of matrix vesicles isolated from the predentin matrix of rabbit incisor tooth organs (Sorgente et al, 1977). The contents of these isolated matrix vesicles have the ability to degrade collagen molecules into two molecular species based upon their molecular weights, like other mammalian collagenases. Sorgente et al (1977) suggested that this activity is involved in the breakdown of the collagen type IV molecules within the basal lamina, which may be the case but in order to show the enzyme activity of these matrix vesicles, type I collagen was used as a substrate. The collagenase of dentin matrix vesicles could be involved in degrading the terminal portions of type I collagen as well as the basal lamina. Release of collagenase may be similar to Katchburian's (1973) proposed

mechanism for leakage and gradual breakdown over time of the plasma membrane of matrix vesicles involved in initial mineralization. It is more likely that the enzymes are released when the plasma membrane is punctured by apatite crystals during the rapid mineralization of predentin. Collagenase can only act on the non-mineralized collagen, and so the only non-mineralized collagen at the enzyme release is the terminal portion. If the collagenase was released prior to this but did not degrade the collagen, it is possible that the enzymes are latent collagenases, which can be activated by enzymes from other matrix vesicles (Eeckout and Vaes, 1977; Sakamoto et al, 1985). Once collagen is degraded it then may be taken up by the ameloblast, along with the basal lamina (BL). Evidence for this uptake may be the appearance of many pinocytotic vesicles along the apical surface of the ameloblast (Fig. 3a; Slavkin and Bringas, 1976). While the collagenase activity is degrading the terminal portions of collagen fibrils, mineral still advances along the fibrils toward the ameloblast, which begin to release enamel matrix. Warshawsky (1985) best described this interaction of the enamel and dentin matrices by saying that the enamel matrix "bathes" the surface of dentin. The interaction between partially mineralized collagen fibrils of dentin and the non-mineralized enamel matrix may be that the frayed terminal portions of collagen fibrils are suspended in the enamel matrix providing an intimate association between these heterotypic matrices where dentin apatite can be presented directly to the enamel matrix.

Selected-area dark field imaging has shown that there is an intimate association between dentin and enamel crystals in both initial and more mature areas of the DEJ (Figs. 6d, and 8b-d). This

direct contact between dentin and enamel suggests enamel's reliance upon dentin for its mineralization, however, further studies may be required to confirm this direct crystal continuity. The determination of an irrefutable crystal relationship between dentin and enamel may prove to be difficult because of the many technical limitations associated with electron microscopy and biological apatite. Some of these limitations include spatial overprojection and truncation of the small crystals within thin sections (Hunziker et al., 1989), limited detection of small crystals or sparsely distributed crystals which have few lattice repeats, and fracture or displacement of the crystals which may occur during sectioning. In addition, apatite crystals are highly susceptible to radiation damage in the electron microscope. This damage is most noticeable in enamel crystals which have a central dark line and/or a holey appearance after only a short time under the electron beam (Kerebel et al, 1979; Warshawsky and Nanci, 1982).

If it is assumed that dentin apatite crystals are exposed to and grow into the enamel matrix, then there must be a difference in the mechanisms by which the matrix controls crystal size. The enamel matrix appears to have a different control mechanism for crystal growth from dentin as dentin crystals which grow into the enamel matrix are not restricted to a uniform size. All other skeletal tissues which contain matrix vesicles and collagen (calcified cartilage, bone, calcified turkey tendon, and dentin) have apatite crystals with approximately the same dimensions of 11- 17 x 5 x 5 nm as determined by SADF, and X-ray diffraction (Arsenault, 1989; Arsenault and Grynpas, 1988; Jensen and Moller, 1948). Initial enamel crystals, although somewhat longer than dentin crystals appear to have

similar widths, and thicknesses (Kerebel et al, 1979), but as maturation occurs the crystals grow until there is little space between them, this unlimited growth can be attributed to the unique enamel matrix (Robinson et al., 1983).

As enamel crystals are intimately associated with dentin crystals, the enamel sheaths which surround enamel crystals appear to be associated with the surfaces of dentin collagen fibrils (Figs. 3c, 5a, b and 9d). There may be several ways in which the sheath is involved in the formation of enamel crystals or the continued growth of dentin crystals. X-ray diffraction studies have demonstrated that the enamelins, a heterogeneous group of glycoproteins that make up the enamel sheath, have a beta pleated sheet conformation which approximates the enamel crystal surface (Glimcher et al., 1961; Jodaikin et al., 1987; 1988; Traub et al., 1985). These studies suggest that the sheath may provide some structural support for the crystal by aligning phosphate groups to be utilized in apatite formation in an organization similar to the unit cell dimensions of the crystal, so that it extends along its c-axis. The sheath may also have an active role in the formation of enamel crystals because it can be remineralized in the presence of calcium chloride, after demineralization (Traub et al., 1985), and electron spectroscopic analysis has demonstrated a co-localization of calcium and phosphate throughout the thickness of the sheath (Arsenault and Robinson, 1989). There are some similarities between the amino acid compositions of the enamelins which make up the enamel sheath and the posphoryns which are thought to have both an inductive and an inhibitory role in dentin mineralization; the major amino acids of both are serine and

aspartate (Termine et al., 1979; Dimuzio and Veis,1978). The enamelins appear not to have the restrictive influence on crystal growth that the phosphoryns exhibit over dentin crystals (Lussi et al., 1988). The continuity that exists between the organic matrices of dentin and enamel may be between collagen-associated phosphoryns and the enamelins of the enamel sheath.

The phenomenon of a crystal sheath has also been observed in invertebrates. Addadi and Weiner (1985) show that acidic matrix macromolecules, extracted from mollusks, formed a beta pleated sheet rich in aspartic acid which interacts with the crystal controlling the pattern and rate of calcite crystal growth. The protein composition of these macromolecules is similar to those of enamelins. It appears that the sheath acts to compartmentalize a growing crystal by providing not only a localized environment for continuous crystal growth, but also in protecting the ameloblast from direct crystal contact (Figs. 4d-f). This last function of the sheath does not appear to be needed in other mineralized tissues because there is always a layer of extracellular matrix between the cells and the mineral.

CONCLUSIONS

Selected-area dark field has shown that there is an intimate relationship between the inorganic matrix components of dentin and enamel, and conventional EM of demineralized sections of the DEJ has demonstrated that this relationship includes the organic matrix components. These results, combined with the information known about

the temporal events of DEJ formation, suggest that the formation of enamel is dependent upon dentin mineralization. If this is the case, then enamel formation must be considered a secondary mineralization process that is initiated by matrix vesicles within predentin. Dentin apatite crystals grow epitaxially to the surface of dentin and then into the enamel matrix where they are bounded by the enamel sheath and take on the characteristics of enamel crystals (Fig. 9d).

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