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Special Review

Dental mineralization

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Introduction

Teeth are composed of the mineralized tissues dentine and cementum, and a mineralized structure, the dental enamel. The pulp may also mineralize in response to aging processes and/or pathologic aggressions (caries, dental treatments). The process of mineralization is specific for each dental tissue. For example, the mineralizations of enamel and dentine are totally different: the former is unique in the body, involving specific proteins not found elsewhere, whereas the latter belong to a rather large group of connective tissue mineralizations involving collagen and non-collagenic matrix proteins. Within dentine, considerable differences exist between mantle and circumpulpal dentines, between intertubular and peritubular dentines. Cementum also displays its own specificity. Mineralization occurs either through cell-derived microstructures: matrix vesicles and/or intracellular mineralizations and cell debris, or cells secrete an unmineralized extracellular matrix which eventually becomes a mineralized structure. In both cases, phosphate and calcium accumulate, combine and stabilize in the form of a more or less pure hydroxyapatite (HA) contaminated by carbonate and magnesium.

In this article, we shall review the various types of mineralization occurring during the formation of each type of dental tissue, with emphasis on dentine and enamel. In spite of the tissue specificities mentioned above, dental tissues constitute important models for gaining insight into the general processes of biological mineralization, and such studies may also shed light on what happens in bone and cartilage. In addition, we are going to focus on the potential role of some matrix components in mineralizing processes, and especially on proteoglycans, phospholipids, and phosphorylated proteins.

Table 1 summarizes the various relationships between organic structures (cells or extracellular matrix components) and the pathway followed specifically by each dental tissue or dental structure which underwent mineralization.

The role of cell-derived microstructures: matrix vesicles and cell debris in dental mineralization

Cell budding or cell fragmentation lead to the formation of matrix vesicles (MV), which are seen at sites of beginning mineralization, such as at the onset of mantle dentine formation and during early stages of cementogenesis. These trilaminated membrane-bound organelles, 30-200 nm in diameter, serve as initial loci of mineralization. They display many specific chemical properties and have a composition which is different from the plasma membrane from which they are derived (see for review Wuthier 1988). MV contain a variety of enzymes including high levels of alkaline phosphatase, pyrophosphatase, adenosine triphosphatase and nucleotide triphosphate pyrophosphohydrolase. These enzymes are responsible for the increased concentration of organic phosphate within the MV. Recently, metalloproteinases which degrade proteoglycans have been identified in MV, whereas such enzymes were not detected in the plasma membrane of chondrocyte-derived fractions (Dean *et al.*, 1992). The phospholipid-dependent Ca^{++} binding protein, annexin II, has been identified as one of the major MV proteins. The composition of phospholipids differs from that of cell plasma membrane because MV are enriched in phosphatidylserine (PS), sphingomyelin (SM), glycosphingolipids and free cholesterol, and are depleted in phosphatidylcholine (PC). Substantial amounts of Ca^{++} and inorganic phosphate are present in MV. Bonucci (1967) and Anderson (1967) were the first to report that in growth cartilage initially octacalcium phosphate (OCP) appears inside such structures. At a later stage, the crystalline mineral displays a more hydroxyapatite (HOP)-like character. The facts that MV are the only structures containing crystals in early stages of mantle dentine formation and formation of initial cementum, and that various stages of crystal growth were revealed by electron diffraction, suggest that they are involved in the mineralization of at least some tissues at some stages of formation (Yamamoto, 1966; Hayashi, 1983, 1985, 1987; Kogaya and Furuhashi, 1988).

Amorphous mineral deposits have been seen to be associated with the plasma membrane of young odontoblasts and chondrocytes (Almuddaris and Dougherty, 1979; Dougherty, 1983). Membrane-associated amorphous deposits of phosphate and calcium have been seen in vesicular structures protruding from the surface of pre- and young odontoblasts. Hence, they may be involved in the formation of MV. Intracellular amorphous granules where phosphate and calcium have also been detected have been observed in mitochondria (Sayegh and Abousy, 1977; Goldberg and Escaig, 1984a). The assumption that only odontoblasts engaged in dentin mineralization display such intramitochondrial granules and that they are not seen when dentine mineralization is advanced, was ruled out when rapid-freezing followed by freeze-substitution was used (Goldberg and Escaig, 1984a). Accumulation in calcium internal stores regulates the calcium level in the cytosol and leads to the formation of such aggregates irrespective of the extracellular stage of mineralization. This regulation is directly under the control of calcium binding proteins of the annexin group and the E-F hand group (Goldberg *et al.*, 1991).

Intracellular accumulation of mineralizing material has been observed in biomaterial-associated calcification inside connective tissue cells (Schoen *et al.*, 1988) and inside cells in an organoid culture system of fetal rat calvaria (Zimmermann *et al.*, 1991). In the extracellular matrix mineralization occurs through cell debris, observed in the vicinity of calcifying nodules in the growing mineralizing material in human semilunar cartilages (Ghadially and Lalonde, 1981), in leg tendons from turkey (Landis, 1986), in osteoblasts in culture (Zimmermann *et al.*, 1991) as well as during ossification of long bone cortices in mouse fetuses (Zimmermann, 1994). Intracellular mineralization, cell necrosis, and the formation of cell debris or vesicles due to cell disruption have also been observed in pulp cell culture (Thonemann *et al.*, 1994) (Figs. 1-3). It is still not known if this occurs only in an *in vitro* system or if it also occurs *in vivo*, for instance when pulp tissue mineralizes in response to aging, caries decay or restorative treatment with dental biomaterials. In such cases, pulp stones or diffuse mineralizations are observed. This reparative dentine or pulpal tissue has osteogenic characteristics (bone-like structures and/or osteo-dentine).

TABLE 1

MINERALIZED DENTAL TISSUES AND STRUCTURES: CELL-DERIVED MICROSTRUCTURES AND EXTRACELLULAR MATRIX COMPONENTS INVOLVED IN BIOMINERALIZATION PROCESSES

	Cell derived or matrix derived structure	Type of dental tissue or dental structure	Cellular and/or extracellular matrix components which might be implicated in biomineralization
Cell-derived microstructures implicated in biomineralization	Matrix vesicles	Mantle dentine Early acellular cementum	*Membrane phospholipids *Proteoglycans *Annexin II
	Intracellular mineralizations- and cell debris	Pulp mineralization *"in vitro"*: -pulpstones or diffuse mineralizat.	*Membrane phospholipids *Proteoglycans
Mineralization induced by extracellular matrix	Extracellular matrix containing both collagenic and non-collagenic components:	*Intertubular circumpulpal dentine	*Collagens: I, I trimer, V, VI. *Phosphorylated proteins: -[dentine phosphoproteins, bone sialoprotein, osteopontin, osteonectin.] *Non-phosphorylated dentine sialoprotein: *Proteoglycans [C4-S C6-S, decorine, biglycan] *Acidic glycoproteins: GLA-protein *Phospholipids
		*Cellular and acellular cementum	*Serum proteins: albumin-α2HS-glycoprotein *Growth factors
	Extracellular matrix containing only non-collagenic components:	Peritubular circumpulpal dentine	*Glycoproteins *Proteoglycans *Lipids *Serum proteins
	"	Enamel	*Amelogenins *Non-amelogenins proteins: -Enamelins (tuftelin) -Tuft proteins *Lipids (cellular and extracellular) *Proteoglycans

Mineralization processes associated with cell-derived microstructures occur mainly during the formation of mantle dentine, initial cementum and *in vitro* pulp cell culture, while the mineralization of the major parts of the hard dental tissues, enamel and dentines, results from the transformation of an extracellular matrix into a calcified tissue. Intertubular dentine results from the mineralization of a complex extracellular matrix containing collagen as the major protein and many non-collagenic phosphorylated and non-phosphorylated proteins, as well as free glycosaminoglycans (GAGs) and lipids. In contrast, enamel does not contain collagen and is a good example of non-collagenic mineralization. Even if cell debris may be present inside forming enamel (Goldberg and Escaig, 1984b), it probably does not play the role of matrix vesicles.

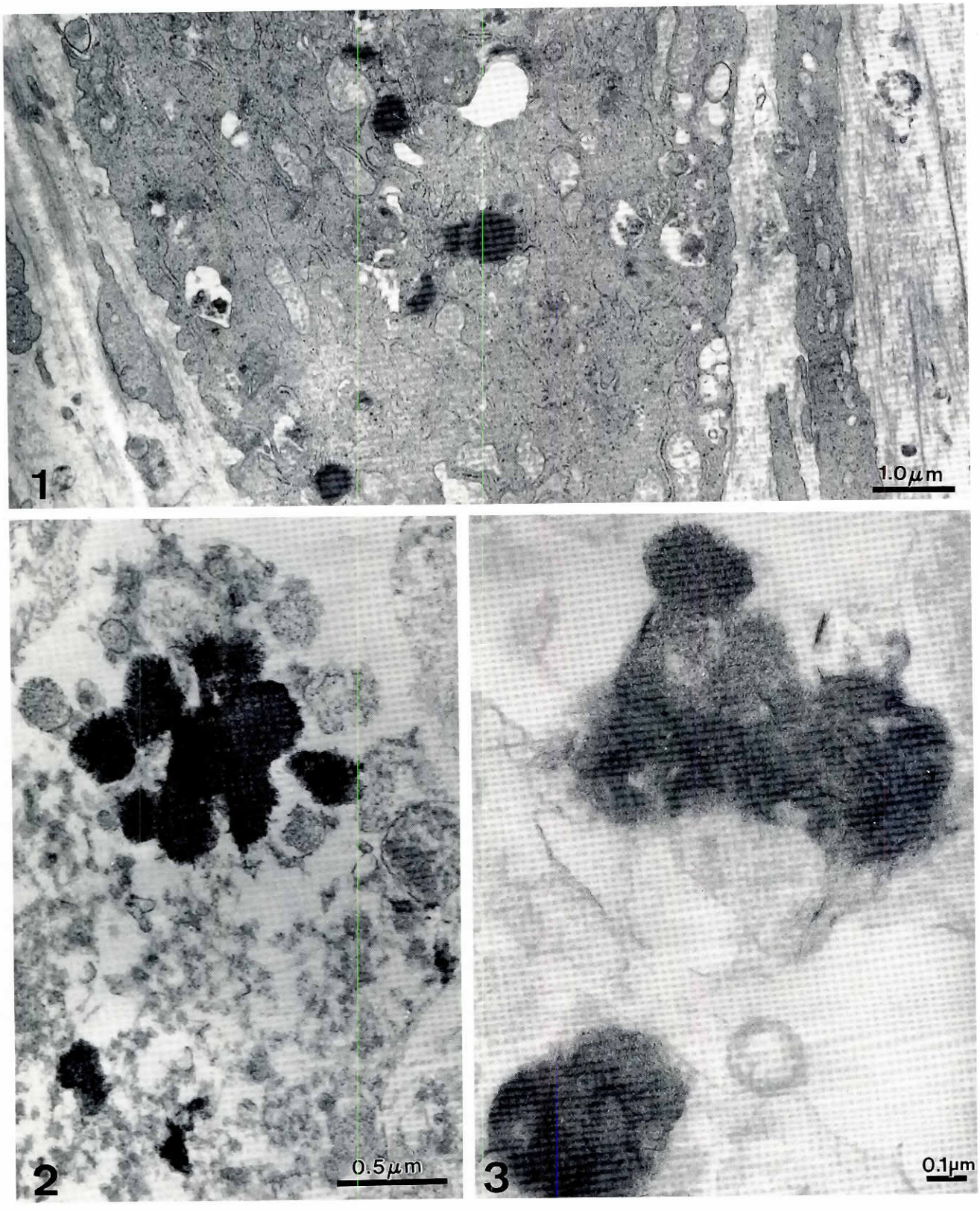
Dentinogenesis: circumpulpal intertubular dentine mineralization

The mechanisms of formation of mineralized tissues, the associated matrix components and their implication in the mineralizing processes, constitute the topic of many reviews (see for example Eastoe, 1968; Fisher and Termine, 1985; Weiner, 1986; Boskey, 1989, 1992). More specifically, reviews related to dentinogenesis, dentine matrix proteins and their potential roles have been published by Leaver *et al.* (1975), Butler (1984), Bronckers *et al.* (1989), Linde (1989), Linde and Goldberg (1993) and Goldberg and Takagi (1993).

From a structural point of view, intertubular dentine results from the synthesis and secretion by odontoblasts of a collagenic matrix constituting the predentine, and its subsequent transformation into dentine (Fig. 4). The cells, which take their origin from the neural crest, form a mono- or bistratified layer of cell bodies, densely packed at the periphery of the pulp. Odontoblast cell bodies are implicated in the synthesis of collagenic and non-collagenic matrix molecules. In the distal part, gap and desmosome-like junctions link together the plasma membranes. There are also a few tight junctions. However, these junctional complexes form a leaky barrier which allows ions, some minor components originating from the blood sera and some pulpal material to diffuse between the cells. This exogenous material is incorporated first into predentine which is then transformed into dentine. However, most of the major dentine components are produced and controlled by odontoblasts. Long processes extend from the cell bodies, cross the predentine and penetrate the dentine inside dentine tubules. These processes, the main trunk and lateral branches, are responsible for the transport and secretion of matrix components. These components are secreted either in the proximal part of predentine (collagen and proteoglycans) or near the mineralization front at the dentine edge (non-collagenic proteins) (Figs. 4,5). Reinternalization of matrix residues (N-terminal non-helical procollagen extensions, degraded proteoglycans) occurs also along odontoblast processes, controlled by coated vesicles.

Dentine composition

Seventy per cent in weight of the dentine is formed by a mineral phase, 20% by an organic matrix and about 10% by water. By volume, the mineral phase accounts for 50%, the organic matrix



Figs. 1-3. Mineralization inside cells and cell-derived microstructures. (1) *Calf pulp cell in culture. Intracellular mineralizations appear as electron-dense round structures with a denser rim at the periphery. Glutaraldehyde/paraformaldehyde fixation, uranyl acetate (UA), lead citrate (LC). x16200.* **(2)** *Calf pulp culture fixed with glutaraldehyde in the presence of CPC. Mineralizing cell debris. UA-LC. x40500.* **(3)** *Calf pulp culture. CPC-glutaraldehyde. Mineralization occurs in the extracellular matrix in relation with filamentous GAGs and cell debris. Needle-like structures develop in electron-dense aggregates. UA-LC. x81000.*

30% and the water phase 20% respectively. This is the general composition of dentine, assuming homogeneity of the tissue. However, it is well documented that considerable variations occur between circumpulpal and mantle dentine as well as between dentine in root and crown.

Collagens

Collagens are the major components of dentine. They constitute 90% of the total dentine matrix, but are absent in peritubular dentine.

Collagen I [$\alpha1(I)_2$, $\alpha2(I)$] is the predominant type present in dentine. Thirty percent of the salt-extractable and 10-15% of the insoluble fraction contain a Type I trimer $[\alpha1(I)]_3$. Atypical forms of collagen fibers appearing as centrosymmetrical structures, resembling segment long spacing (SLS) crystallites, have been observed in the predentine of normal rats (Warshawsky, 1972; Weinstock, 1972), after vinblastine injection (Miake and Takuma, 1985) and in surgically induced hypoparathyroidism/hypothyroidism (Acevedo *et al.*, 1995). The presence of type III collagen is more controversial. It seems to be absent in normal predentine and dentine, but is present in some pathological conditions, such as hereditary opalescent dentine. Type V has been identified in odontoblast cultures and tooth germs and accounts for only 3% of the collagen synthesized by odontoblasts and secreted in predentine. Type IV is present only at the onset of dentinogenesis, when a basement membrane isolates the first layer of unmineralized dentine from presecretory ameloblasts. Type VI has been identified in predentine as a minor component.

Many investigations performed before 1980 have identified several non-collagenous components in predentine and dentine (see for review Leaver *et al.*, 1975). They included glycosaminoglycans in the form of proteoglycans, acidic glycoproteins, phosphoproteins and proteins originating from the blood sera. In addition, lipids have also been found (Prout *et al.*, 1973). In 1980, Linde *et al.* extracted non-collagenous proteins (NCP) from dentine with 4M guanidine hydrochloride containing enzyme inhibitors. Prior to demineralization, they found only a proteoglycan fraction. After demineralization with an EDTA solution containing protease inhibitors, 4 types of macromolecules were identified in the extract: acidic glycoproteins, γ-carboxyglutamic acid (Gla)-containing proteins, phosphoproteins and proteoglycans. This list does not include some other matrix proteins and growth factors which need additional procedures to be solubilized, proteins originating from the sera, and various form of lipids.

Non-collagenic proteins (NCP) and other matrix components in dentine

Non-collagenic proteins (NCP) and other matrix components in dentine include:

Dentine phosphoproteins (DPP)

Phosphoproteins are the major group of dentine NCP (more than 50%). Depending on the species, several types of phosphoproteins have been extracted:

- highly phosphorylated phosphoprotein (containing about 46% phosphoserine and 45% aspartic acid in bovine teeth and >40% phosphoserine in rat incisor), referred to by DiMuzio and Veis (1978) as phosphophoryn,
- moderately phosphorylated (25% phosphoserine) phosphoproteins
- and weakly phosphorylated (5-7% phosphoserine) phosphoproteins.

Phosphophoryn has a Mr of about 155 000 and a ß-pleated sheet conformation.

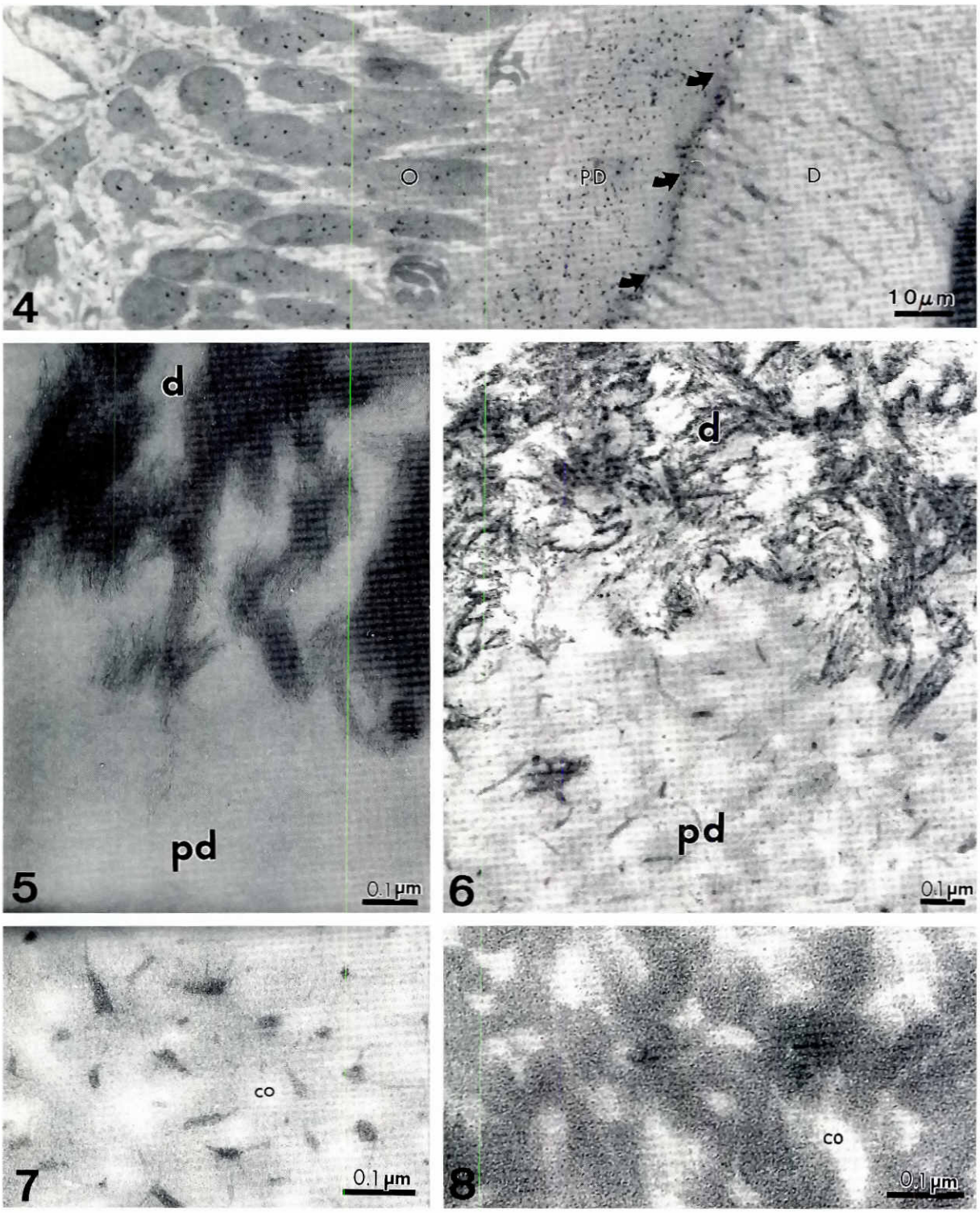
From the work published by Weinstock and Leblond (1973) it appears that (^{33}P) phosphate and (^{3}H) serine are incorporated into odontoblasts and secreted at the predentine/dentine junction within 1 to 2 h after injection. This pathway was further confirmed by Goldberg *et al.* (1987a) and Inage and Toda (1988). Using Stains All staining, Takagi *et al.* (1986) found that phosphophoryn is present in all the mineralized dentine except the first external layer of mantle dentine. Predentine remains unstained, as well as pulp cells. It is interesting to note that root dentine only contains half the amount of DPP present in the crown (Takagi *et al.*, 1988). No staining was seen in secondary and reparative dentine (Takagi and Sasaki, 1986) nor in Dentinogenesis Imperfecta Type I and II (Takagi *et al.*, 1983; Takagi and Sasaki, 1988). The same distribution was found using immunocytochemical technique (MacDougall *et al.*, 1985; Nakamura *et al.*, 1985; Gorter de Vries *et al.*, 1986; Rahima *et al.*, 1988) and phosphotungstic acid-chromic acid staining (Goldberg *et al.*, 1978) (Figs. 5,9 and 11). Because it is present in dentine and not in predentine, DPP are believed to act as mineral nucleators. Depending on concentration, *in vitro* DPP can either promote or inhibit hydroxyapatite formation.

It has also been reported that phosphoproteins retard the rate at which collagen molecules undergo self-assembly into fibrils after a relatively short time (~4 h) (Gelman *et al.*, 1980). When DPP are introduced during type I collagen fibrillation after a much longer time (5 days), the fibrils formed are significantly wider than in the absence of DPP (Clarkson *et al.*, 1993). In developing bone, the NH_2-terminal propeptide of the $\alpha1$ chain of type I collagen is a phosphoprotein of Mr 24 kDa (Fisher *et al.*, 1987). It is unknown if this finding applies to dentine.

Since dentine phosphoproteins act as extracellular calcium-binding proteins, their binding properties with collagen and Ca^{2+} have been investigated. DPP has a very strong Ca^{2+} affinity with two sets of binding sites, at an optimum pH of 8.2 (Zanetti *et al.*, 1981). When *in vitro* precipitation of calcium phosphate was carried out by one-dimensional double diffusion system in agar gel, the precipitation band was enhanced in presence of DPP. However, crystal growth was decreased. Thus, DPP allows the formation of new nuclei, but inhibits the growth of the already formed crystals. Therefore, Fujisawa *et al.* (1987) concluded that the possible role of DPP may be the regulation of crystal growth behind the mineralization front. In an *in vitro* gelatin gel diffusion system, phosphophoryn in low concentration promoted the formation of hydroxyapatite. At higher concentration, the growth of hydroxyapatite was inhibited, suggesting inhibition of secondary nucleation (Boskey *et al.*, 1990). DPP attached to a surface induces mineral formation *in vitro*, whereas when free in solution, DPP acts as a mineral inhibitor (Lussi *et al.*, 1988).

Other phosphorylated proteins

Osteonectin (ON)/SPARC or BM 40 is a phosphorylated glycoprotein, which has an EF hand structure, inhibits HA growth, but promotes Ca and P binding to denatured collagen. ON is virtually absent in rat dentine, but present in porcine dentine, where intense dentine staining was seen in unerupted teeth. In bovine dentine, ON constitutes 4-6% of the total protein extractable from the tissue. In erupted porcine teeth, anti-ON staining in dentine was concentrated around dentinal tubules, was weaker in the cells of the pulp, and intense in odontoblasts (Tung *et al.*, 1985). In human teeth, the predentine, odontoblasts and their cell processes show



Figs. 4-8. Circumpulpal dentine formation — visualization of phosphoproteins and proteoglycans. (4) *Semithin section of rat incisor. The rat was injected with ^{3}H-serine 4 h, then killed and the tissues processed for autoradiography. Labeling is seen over pulp cells (P), odontoblast cell bodies (O), the whole thickness of predentine (PD), and at the mineralization front (arrows), but not in dentine (D). x1000.* **(5)** *Unstained section of the mineralization front, at the junction between predentine (pd) and dentine (d). Crystallites appear and develop along collagen fibrils. x110000.* **(6)** *Cuprolinic blue-aldehyde fixation in presence of 0.3 M $MgCl_2$. GAG/cationic dye aggregates are seen in predentine (pd) between collagen fibrils. They are located at the surface of groups of collagen fibers in dentine (d). There is some staining at the junction between predentine and dentine where mineralization occurs. x81000.* **(7)** *Cuprolinic blue-aldehyde fixation. Rat incisor. Aggregates with radiating filaments are seen in predentine. The collagen fibers (co) appear as electron-lucent areas. x140000.* **(8)** *After rapid-freezing and freeze-substitution, GAGs in predentine stained with Alcian blue appear as an expanded amorphous gel between electron-lucent collagen fibers (co). x140000.*

a strong staining reaction (Reichert *et al.*, 1992). *In situ* hybridization studies with mRNA show an intense reaction over the odontoblasts.

Osteopontin (OPN), also called *bone sialoprotein I (BSP I),* is a 44 kDa bone phosphoprotein also found in dentine. It possesses mostly an α-helical structure, with a Ca binding loop rich in aspartic acid. Immunostaining reveals faint staining in some young odontoblasts before mineralization of the dentine, but no staining in preodontoblasts, mature odontoblasts, and dentine. This phosphorylated glycoprotein is rich in aspartic acid, serine and glutamic acid and contains 12 phosphoserine. Its sialic acid content varies according to species between 4.8 and 10.4%. An Arg-Gly-Asp sequence, or RGD, is probably involved in attachment and spreading of osteoblasts and fibroblasts *in vitro* by binding to integrins. Osteopontin and/or its mRNA are present in odontoblasts. As a phosphorylated protein, osteopontin may promote the formation of mineral in dentine.

The phosphorylated sialoproteins or Bone Sialoprotein (BSP), also called *bone sialoprotein II,* is a glycoprotein with a Mr 70 000-80 000 and ß-pleated sheet. This molecule contains 12-18% sialic acid, depending on the species, 7% glucosamine, and 6% galactosamine. It contains sulfate, present principally as tyrosine sulfate with about 30% attached to O-linked and N-linked oligosaccharides. Because it contains keratan sulfate chains in rabbit bone, it is also a proteoglycan. Therefore, BSP stains with Alcian blue and Stains All. Rat BSP also contains a RGD sequence, and therefore, like OPN, promotes attachment and spreading of the cells, but to a lesser extent than OPN. BSP enhance type I collagen fibrillogenesis. Small amounts of BSB have been found in dentine. At early stages of development, *in situ* hybridization with cRNA probes does not reveal any labeling, whereas at day 21 the mandibular incisors of the rat fetus display intense labeling in the odontoblasts. Therefore, this protein may mediate the initial stage of mineralization (Chen *et al.*, 1992). Immunoreactivity was seen in odontoblasts and their processes, and in the peritubular dentine of porcine dental tissues (Chen *et al.*, 1993).

Non-phosphorylated sialoprotein

Dentine sialoprotein (DSP), was initially called 95 k glycoprotein and more recently *53-kDa DSP.* This is a sialic acid-rich glycoprotein. Like the other sialoproteins, DSP is rich in aspartic acid, glutamic acid, serine and glycine, but contains no cycteine nor phosphate. The carbohydrate content is high (30%) and includes 9% sialic acid. Several N-glycosides and O-glycosides are present. Synthesized by odontoblasts, the molecule is secreted into predentine and dentine. These molecules appear within young odontoblasts before the mineralization of dentine. Immunostaining is intense in odontoblast processes. Early predentine stains intensively, whereas the staining is less intense and restricted to odontoblast processes at more mature stages. The cells and matrix of the dental pulp are also stained (Butler *et al.*, 1992; D'Souza *et al.*, 1992; Bronckers *et al.*, 1993). These molecule, unlike other sialoproteins, have no attachment properties.

Dentine Gla protein (DGP) or Osteocalcin (OC)

This protein is identical to bone γ-carboxyglutamic acid containing protein, GLA-protein. At least 4 closely related fractions have been identified in rat dentine (Linde *et al.*, 1982). This molecule has no effect on mineral formation but retards HA growth. Immunocytochemical data in rats indicate intense staining in odontoblasts and dentine where it was seen to be either restricted to the odontoblast processes (Bronckers *et al.*, 1987), or entirely labeled. The various reports all agree that only very weak staining is seen in predentine (Camarda *et al.*, 1987; Gorter de Vries *et al.*, 1988). In human and bovine teeth, no staining is seen in odontoblasts and dentine. Only the mantle dentine reacts with anti-OC.

Proteoglycans (PGs)

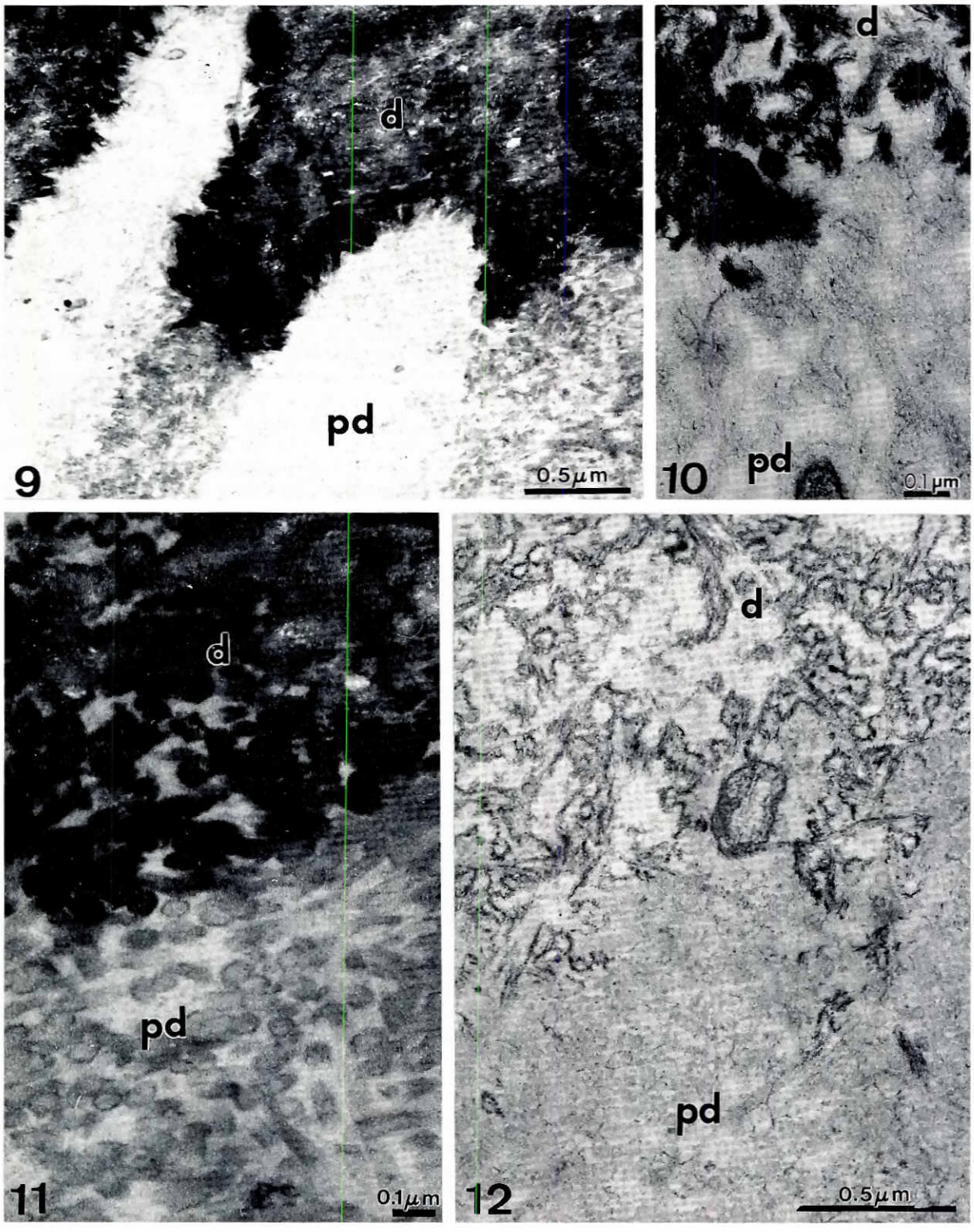
Identified in dentine by Pincus (1950), chondroitin sulfates (C4-S and C6-S) are the major glycosaminoglycans(GAGs) of the tissue (Jones and Leaver, 1974). Utilizing techniques to avoid artifactual degradation and losses, Linde *et al.* (1980) showed that prior to demineralization, a proteoglycan fraction is released with 4M guanidine hydrochloride. Another fraction is released only after EDTA demineralization, together with non-collagenic proteins. It was further demonstrated that the extract from non-demineralized rat incisor contains two groups of PGs: Pd-PG I and Pd-PG II., whereas only one fraction (D-PG) is obtained after EDTA demineralization (Rahemtulla *et al.*, 1984). Pd-PG I have a large molecular weight with large GAG chains composed of C4-S and C6-S isomers. Pd-PG II and D-PG (Mr 70000-120000) have smaller GAG chains composed exclusively of C4-S chains. All these PGs are unable to interact with hyaluronic acid. Heparan sulfate (HS) and keratan sulfate (KS) have also been identified as minor components. However, this is still a matter of controversy.

Small proteoglycans, such as decorine and biglycan have been identified in mineralized tissues. Immunohistochemical data support that C4-S and decorine are present in dentine, whereas C4-S, C6-S, KS, versican and decorine are present in predentine (Takagi *et al.*, 1990).

Electron histochemical investigations with cationic dyes, cationic detergents and hyaluronidase gold complexes allow visualization of PGs in predentine in the spaces located between the collagenic network. They are homogeneously distributed throughout the whole thickness of predentine. In dentine, PGs appear as needle-like structures or crystal-ghosts associated with the surface of collagen fibers (Figs. 6 and 7). Rapid freezing followed by freeze substitution evidences that PGs are present as an amorphous gel in predentine, and fill all empty spaces between the collagen fibrils (Fig. 8). These latter are transported from the place where they are secreted (the proximal predentine at the junction between cell bodies and predentine) toward the distal part of predentine (the mineralization front at the dentine edge). From this organization, and experiments using inhibitors of secretion (vinblastine sulfate), it was concluded that PGs are among the major components of the so-called "ground substance" and provide an adequate medium for transports and diffusions (Goldberg *et al.*, 1987a). Other observations support the claim that PGs share the space between collagen fibers with phospholipids.

In contrast with the amorphous structure seen in predentine, PGs in dentine are closely associated with the surface of collagen fibers or groups of collagen fibres. The structure appears either as dotted lines or needle-like structures. Again, they share these properties with phospholipids. It may be concluded that these structures include PGs, phospholipids and a proteinaceous material, recognized also in other calcifying tissues (Bonucci, 1987).

It has long been known that there is a loss of PGs at sites where bone mineralization is initiated (Baylink *et al.*, 1972). *In vitro* studies provide evidence that hydroxyapatite formation in collagen gels is inhibited by chondroitin sulfate in solution (Blumenthal *et al.*, 1979; Chen *et al.*, 1984; Chen and Boskey, 1985; Hunter *et al.*, 1985). However, *in vivo*, PGs may function as a promoter of mineraliza-



Figs. 9-12. Phosphorylated proteins and phospholipids in predentine and dentine. (9) *Glutaraldehyde-osmium tetroxide fixed section stained with phosphotungstic acid in chromic acid. Rat molar. Predentine (pd) is unstained. The junction between predentine and dentine is densely stained. Dentine (d) is stained but to a lesser degree. x40000.* **(10)** *Malachite green-aldehyde fixation. Phospholipids are seen as filaments in predentine (pd) in spaces between collagen fibers. In this undemineralized section, only crystallites are seen in dentine (d). LC. x81000.* **(11)** *Phosphotungstic acid staining. In predentine (pd) a thin ring is seen around unstained collagen fibers. In dentine (d), the staining around and inside collagen fibers is dense. Phosphoproteins are stained with this method. x110000.* **(12)** *Malachite green-aldehyde fixed tissue. EDTA-demineralized section. Phospholipids appear as electron-dense aggregates in intercollagen spaces in predentine (pd) and as needle-like structures at the surface of groups of collagen fibers in dentine (d). LC. x60000.*

tion, acting as a cation-exchanging calcium reservoir (Hunter, 1991). When immobilized on a solid support, PGs induce hydroxyapatite formation whereas in solution they inhibit hydroxyapatite formation and growth (Linde *et al.*, 1989).

The differential distribution recognized on sections may correspond to such different functions. In predentine, as an amorphous hydrated gel, PGs may play a role in transport and diffusion and act as HA inhibitors. In contrast, in dentine, PGs adsorbed on collagen surfaces promote HA initiation.

Serum proteins

Albumin has been detected in dentine. An autoradiographic investigation using labeled albumin showed incorporation of the precursor into predentine (after diffusion between the odontoblasts), and eventually the labeling was seen in dentine (Kinoshita, 1979).

α_2HS glycoprotein, a plasma protein with a molecular weight of about 50 kDa, has been detected in dentine. Immunolocalization in bovine and human teeth evidenced the presence of the molecule in peritubular dentine (Takagi *et al.*, 1990).

Lipids

As shown in Table 2, in bovine teeth the distribution of phospholipids varies between predentine and dentine (data from Ellingson *et al.*, 1977 and Shapiro *et al.*, 1966).

From this comparison, it comes out that some classes of phospholipids are associated with the mineral phase and therefore may be involved in dentine biomineralization. Although there are many technical difficulties which have to be overcome before any visualization of lipids may be carried out, we have shown:

- that phospholipids, visualized with malachite green aldehyde (Goldberg and Septier, 1985), with malachite green-acrolein-osmium tetroxide freeze substitution after rapid freezing (Goldberg and Escaig, 1987), and with iodoplatinate (Vermelin *et al.*, 1994) are located in inter-collagen spaces in predentine, whereas they are located along individual fibers or at the surface of groups of collagen fibres in dentine (Figs. 10,12).
- that phospholipase-gold complexes yield increased labeling in the distal part of predentine close to the predentine/dentine junction (Goldberg *et al.*, 1994)
- that the distribution of phospholipids and glycosaminoglycans in predentine and dentine is very similar, as shown by visualization with cationic dyes, cationic detergent, enzyme digestion and enzyme-gold complex labeling (Goldberg and Takagi, 1993). However, GAGs are homogeneously distributed in predentine and are increased near the mineralization front. In dentine also, crystal ghosts or needle-like structures stain both for phospholipids and GAGs. This dual distribution contributes to the properties of the tissue and may play a role in biomineralization.

This provides evidence that PGs are not involved in the increased Ca^{2+} level detected by EELS in the distal predentine compared to the proximal and central predentine (Plate *et al.*, 1992). In contrast, phospholipids may be responsible for the higher calcium content in the distal predentine, before any visible precipitation at the mineralization front.

This dual distribution is now under investigation in our laboratory.

Growth factors

Growth factors IGF-I, SGF/IGF II and TGF-ß have been identified in dentine (Finkelman *et al.*, 1990). Their role in dentine mineralization is so far unknown. They may act as cytokines or are simply fossilized during dentine formation.

TABLE 2

DISTRIBUTION OF PHOSPHOLIPIDS IN PREDENTINE AND DENTINE

	Phospholipids in predentine	Phospholipids extracted from non- demineralized dentine	Phospholipids extracted from demineralized dentine
PC	56%	67.1-77.2%	11.6-18.2%
PE	23.4%	12.5-20.4%	28.7-37.1%
PS	5.2%		18.1-35.2%
PI	5.3%		
Sphingomyelin	5.2%	9-11.3%	14.4-18.2%

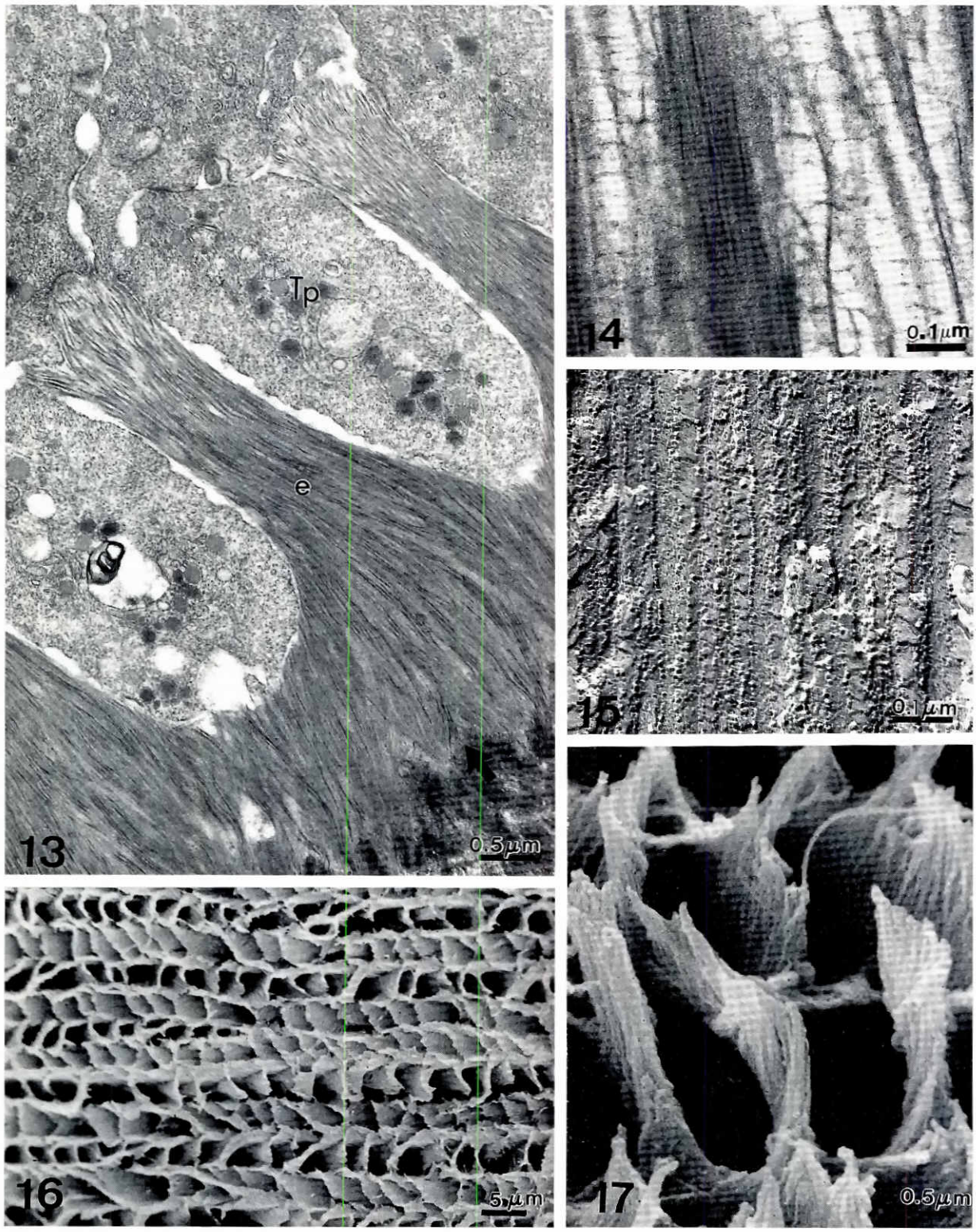
Deposition of calcium

According to Munhoz and Leblond (1974), as soon as 30 sec after intravenous injection of ^{45}Ca, a band of reaction is seen along the edge of dentine. Using the same precursor, Nagai and Frank (1974) showed two transfer pathways: one intracellular and the other intercellular. Five min after intravenous injection the labeling was higher in predentine than in dentine, but more equal after 30 min. After 1 h, it was higher in dentine. Lundgren *et al.* (1994) found that the dentine was labeled 10 min after isotope administration. Odontoblasts were labeled 2 min after intravenous injection of ^{44}Ca. After 5 min, predentine was labeled and the grain density over dentine started to increase. The dentine was labeled densely after 10 min. This favors the opinion that odontoblasts are involved in calcium transport.

There are many arguments in favor of intracellular transport processes. The demonstration of Ca^{2+}-activated ATPase, Na^+/Ca^{2+}-exchanger provides evidence for the existence of calcium channels (see for review Linde and Goldberg, 1993). Calcium binding proteins of the E-F hand family and of the annexin family have been detected in odontoblasts (Goldberg *et al.*, 1987b,1991). This proves at least that many cellular functions are regulated by theses proteins, calcium being dislodged from calcium internal stores toward the cytosol and vice versa. Other cellular regulations imply the presence of calcium and calcium binding proteins: regulation of the cytoskeletal protein synthesis, regulation of exocytosis and endocytosis with the cytoplasmic plasma membrane. How these phenomena are related to calcium diffusion towards the mineralization front is still an unsolved question.

Dentinogenesis: peritubular dentine formation

In circumpulpal dentine, surrounding the dentinal tubules, peritubular dentine is found in many species, including man. This is an example of connective tissue mineralization apparently without collagen fibers. Up to now, only non-collagenic components have been identified. The mineral phase is constituted by isodiametric crystals, 25 nm in diameter, enriched in Mg and carbonates, or crystals 36 nm in length, 25 nm in width and 9.76 nm thick (Schroeder and Frank, 1985). The amorphous organic matrix consists of glycoproteins, proteoglycans and lipids. SEM examination of horse molars reveals that lateral branches of odontoblast processes penetrate minute tubules throughout the whole thickness of the peritubular dentine. This suggests that the formation of peritubular dentine occurs only in the presence of living cells (Goldberg *et al.*, 1980). Although little is known about the mode of formation of peritubular dentine, the presence of osteonectin, bone sialoprotein, osteocalcin and α2 HS glycoprotein indicates that the



Figs. 13-17. Enamel formation. (13) *Tomes' processes of secretory ameloblasts (Tp). Rat incisor. The forming interrod enamel (e) appears as a fibrous structure. The dentine-enamel junction (arrow) is seen near inner aprismatic enamel. UA-LC x20000.* **(14)** *Rat incisor. After rapid-freezing and freeze-substitution, the forming enamel appears as a ladder-like structure. Thin filaments bridge fibers, 30-50 nm thick, where crystallites develop. UA-LC x140000.* **(15)** *Rat incisor. Fixed material, glycerol protected, replica of freeze-fractured forming enamel. Alignments of particles are seen along fiber-like structures. x110000.* **(16)** *Scanning electron microscopy. Anorganic preparation of forming enamel of rat incisor. Alignments of interrod enamel. Empty alveoli will later be filled with rod enamel. x1600.* **(17)** *Anorganic preparation of forming rat incisor enamel seen with the SEM. Fibrous structures are seen in the interrod enamel. x12000.*

matrix results from a secretion by odontoblasts together with diffusion of plasma proteins along the plasma membrane of the processes. Evidence that it is a physiological structure and not a result of degeneration also comes from the fact that in at least two species, elephant and opossum, peritubular dentine is formed at the mineralization front before intertubular dentine (Boyde and Jones, 1972; Acevedo and Goldberg, 1987).

Amelogenesis, enamel formation

The cells of the inner enamel epithelium of the enamel organ differentiate into preameloblasts. The cells then become post-mitotic and are as presecretory ameloblasts involved:

1 in the synthesis of components of the basement membrane which separates epithelial cells from the subjacent mesenchyme (the embryonic dental pulp), and at later stages, of catalytic metallo-enzymes disrupting the basement membrane (Denbesten *et al.*, 1989);
2 in the synthesis of enamel proteins identified by some authors as contaminants of the early non-mineralized dentine (Slavkin *et al.*, 1988).

The first deposition of enamel occurs at the surface of a thin layer of mineralizing mantle-dentine as the presecretory ameloblasts become secretory ameloblasts. The first layer of the forming enamel is aprismatic. Partial degradation of the enamel matrix proteins, due to amelogeninases or the enzyme-like activity of initial crystals, leads to the formation of an amorphous osmiophilic or stippled material in the distal part of the cells, beneath the terminal bars. This leads to the formation of Tome's processes and subsequently to the deposition of interrod material surrounding the processes. The presence of strands of tight junctions indicates the existence of a tightly sealed permeability barrier, which prevents material from passing between secretory ameloblasts (Fig. 13). This configuration provides distinct compartmentalized microenvironment. At this stage, some organic material fills the space left by the ameloblasts as they move away form the dentine-enamel junction (Figs. 16 and 17). More than likely, Tomes' process membranes, which are gradually enriched by cholesterol, split up into small vesicles (Goldberg and Escaig 1984b). When the formation of enamel reaches a certain thickness, in rat Tomes' processes becomes gradually thinner during outer enamel formation. Consequently, the volume occupied by interrod enamel increases at the expense of enamel rods. Gradually, Tomes' processes disappear and the outer last deposited enamel is aprismatic. Then ameloblasts become post-secretory and are involved in enamel maturation. Modulation between smooth and ruffle-ended post-secretory ameloblasts allows the mineral charge to increase, whereas most of the matrix proteins are destroyed and/or removed (Smith *et al.*, 1987). Part of the organic matrix persists in maturing and mature enamel. A newly synthesized basement membrane participated in the control of the transports in and out of the maturing enamel. Finally, post-secretory ameloblasts are reduced in height and either participate in the formation of the Nasmyth's cuticle, a remnant of the enamel organ in teeth of limited growth, or, in the continuously growing rat incisor, merge with keratinocytes of the junctional epithelium and disappear in the cell stream of the oral mucosa. Mature enamel deprived of cells is therefore a mineralized structure, but not a tissue in the strict sense of the word.

During this evolution, many proteins are secreted and recent findings have shed light on a complex situation.

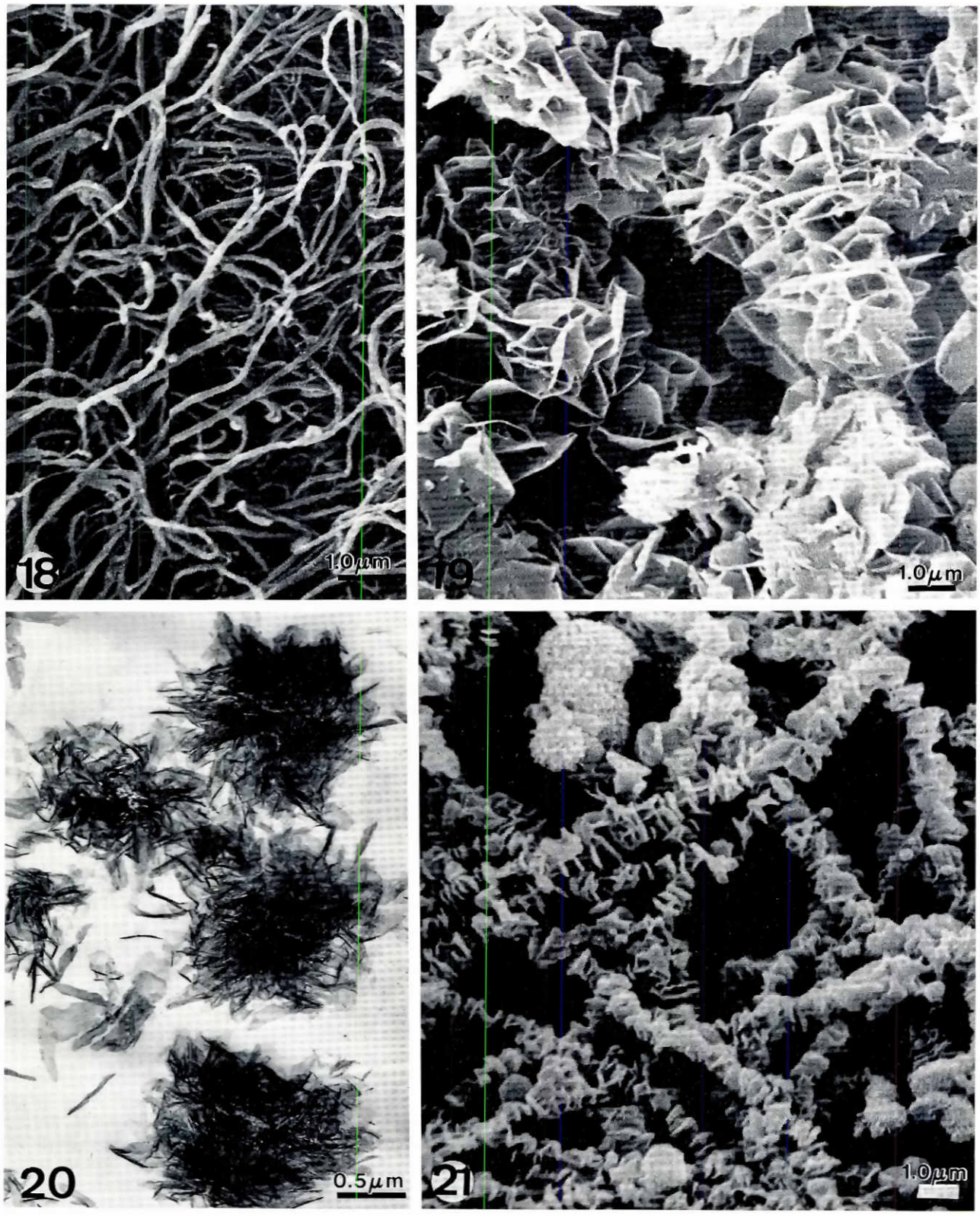
Two groups of enamel proteins were initially identified:

- first, *amelogenins*, proteins insoluble in neutral solutions of 0.5 M EDTA, detected in immature enamel and characterized by their high concentration of proline, glutamic acid, leucine and histidine;
- second, *enamelins*, proteins soluble in 0.5 M EDTA detected in fully mature enamel and characterized by their high concentration of aspartic and glutamic acids, serine and glycine. These latter, detected also in developing unerupted teeth, are members of the *non-amelogenin* protein family.

During maturation, quantitative and qualitative differences are detected, amelogenins being gradually lost, whereas enamelins become the major constituent, associated with the mineral phase.

Amelogenins are hydrophobic molecules. They constitute about 90% of the matrix proteins in the forming enamel. They are low -Mr proteins (initially 27 and 25 kDa) and are degraded rapidly (~5 kDa). Their diversity results from alternative splicing of the mRNA and post-translational modifications. Sequence data have revealed the existence of two distinct amelogenin molecules, identical for the first 33 amino acids, but which differed completely for the remaining 12/13 residues: the tyrosine-rich amelogenin polypeptide (TRAP) and the leucine-rich amelogenin polypeptide (LRAP). These two molecules seem to be derived from a full length amelogenin through a site-specific amelogenin protease. In human, both the X and Y chromosomes carry a copy of the gene, although expression is dominated by the X chromosome. Amelogenins are glycosylated and phosphorylated proteins. Immediately after secretion, amelogenins are submitted to extracellular proteolytic processing. In this context, amelogeninases have been identified (see for review Fincham *et al.*, 1992). Amelogenins are selectively lost during the process of mineralization. Amelogenin functions remain unknown. It has been suggested that these molecules exhibit crystal-growth inhibition activity (Doi *et al.*, 1984). Further investigations have demonstrated that the originally secreted amelogenins (25 kDa) as well as the higher molecular weight components (60-90 kDa) show strong affinity for hydroxyapatite. In contrast, the partially degraded product (20 kDa) loses its adsorption affinity onto hydroxyapatite (Aoba *et al.*, 1987).

Enamelins and/or non-amelogenin proteins are larger proteins with masses up to 70 kDa (Termine *et al.*, 1980). These molecules are hydrophilic and glycosylated. They are crystal-bound proteins. They comprise a heterogeneous population of proteins, as also was the case for amelogenins. For example, in developing bovine enamel, Ogata *et al.* (1988) identified four distinct enamelins having molecular weights of 70, 45, 30 and 28 kDa. In porcine immature enamel, enamelins having molecular weight of 32, 89 and 142 kDa have been isolated, the former is more than likely derived from the latter (Fukae and Tanabe, 1987). Deutsch *et al.* (1991) have identified a novel acidic enamel protein, containing 389 amino acids and with a calculated molecular weight of 44 kDa, which they called "tuftelin". This molecule is similar to tuft protein , a molecule in the Mr range 50-70 kDa (Robinson *et al.*, 1975, 1989). The finding by Limeback *et al.* (1989) that serum albumin dominates preparations of mineral-bound enamel proteins adds to the confusion. This was further confirmed by Strawich and Glimcher (1990) and Strawich *et al.* (1993), who established that albumin accounted for 70-80% of the protein content of the enamelin extract. They identified also α-2 HS glycoprotein, γ-globulin, fetuin and a proline-rich salivary protein. Therefore, they suggest that the term of enamelin is restricted to the protein and peptide components remaining in the enamel of mature teeth and use the term



Figs. 18-21. *In vitro* mineralization. (18) *Collagen gel without calcium seen with the SEM. x8000.* **(19)** *When calcium chloride is allowed to diffuse in the collagen gel, flower-like structures appear in spaces between collagen fibers. x9000.* **(20)** *Anhydrous preparation of the same preparation seen with the TEM. Petal-like structures seen with the SEM appear now as crystallites forming nodules. x27000.* **(21)** *When chondroitin sulfate is added to collagen during gelification, in the presence of calcium chloride, alignments of crystallites are seen along the collagen fibers forming garlands, and only a few round mineral precipitations are located in intercollagen spaces. x7000.*

non-amelogenin as a general term for matrix components genetically distinct from amelogenins, and which are found in developing and mature enamel .

In spite of these conflicting views, and although it is very unlikely that albumin and serum proteins could contribute to the formation of enamel, a few reports shed light on the possible relationships between matrix proteins and enamel mineralization. In this context, using ^{13}C-^{1}H magnetic double-resonance techniques, about 70% of the protein in fetal bovine enamel exhibits rapid isotropic mobility, while the other 30% appears to undergo only spatially restricted, anisotropic motion (Termine and Torchia, 1980). Using X-ray diffraction and Fourier transform infrared spectroscopy, Jodaikin *et al.* (1987) have shown that the acidic enamelins are in the ß-sheet conformation. In contrast, the amelogenins do not show any identifiable regular conformation. These findings, and immunocytochemical investigations (Hayashi *et al.*, 1986; Uchida *et al.*, 1991) support a specific distribution of these proteins in forming enamel. Amelogenins are located in intercrystalline regions, whereas enamelins are part of the crystal ghosts of immature enamel. Gradients of distribution were visualized throughout the forming enamel with anti-25 kDa amelogenin, decreasing from the surface toward the inner part where immunoreactivity is concentrated over the prism sheaths. These latter are also intensely stained by the antisera to 13-17 kDa non-amelogenins in pigs, but not in rats. The 89 kDa enamelin immunoreactivity apparently display the same gradient but no staining of the sheaths. This is also in favor of the concept that enamel proteins soon after secretion are degraded, yielding segments with small molecular masses. Hence, some enamel proteins act as inhibitors of mineralization and may be modulated through their degradation, whereas there is still no definitive proof that some other components act as nucleators.

Analysis of forming enamel have identified a carbohydrate moiety containing L-fucose, D-mannose, glucouronic acid, N-acetyl-glucosamine, N-acetyl-galactosamine, and N-acetyl-mannosamine (Elwood and Apostopoulos, 1975). They are bound to the proteins. Enamelins contain sialic acid, galactosamine and glucosamine. They are significantly different from amelogenins in this respect (Termine *et al.*, 1980). This was further confirmed using soybean agglutinin-gold complexes (Hayashi, 1989) and other lectins (Nanci *et al.*, 1989). Lectins do not bind to porcine amelogenins, while a large number of non-amelogenins are stained with WGA, Con A and MPA lectins. In this respect, two groups of non-amelogenins display distinct properties. WGA and ConA- bind to moieties of the 60-90 kDa glycoproteins whereas MPA binds to moieties of the 13-17 kDa glycosylated proteins. The former are concentrated in the region adjacent to the cells and disappear in the earlier secreted enamel. These molecules are therefore subjected to specific proteolysis and display high adsorption affinity to the apatite crystals. The latter are present throughout the forming enamel. They show only marginal adsorption onto apatitic surfaces. Heterogeneity of enamel proteins is therefore related to variations in carbohydrate moieties (Akita *et al.*, 1992).

Most of the carbohydrates are associated to proteins in the form of glycoproteins. There is also some evidence that some may be present in the forming tissue as glycosaminoglycans or proteoglycans. Autoradiographic investigations using ^{35}S-sodium sulfate (Blumen and Merzel, 1976), ultrastructural histochemistry (Goldberg and Septier, 1986), hyaluronidase-gold complexes labeling (Chardin *et al.*, 1990) support such a possibility. Possibly, GAGs or PGs are involved in the process of elongation of fiber-like structures in the forming enamel, but not in the mineralization process itself.

Lipids are also present in the forming enamel. They account for half the organic matrix. Irving (1959) stated that Sudan black only stained mineralizing enamel whereas the forming enamel remained unstained. It was further demonstrated that pretreatment with chloroform/methanol, a potent lipid solvent, or with phospholipase did not abolish the staining (Patterson *et al.*, 1977). Therefore, the staining is not specific for lipids and stains mostly proteins having lipid-like properties. Autoradiographic investigations using rapid freezing and freeze substitution and ^{3}H palmitic acid as precursor visualized diffuse labeling throughout the whole thickness of forming enamel and not at the sites where mineralization is supposed to occur. This diffusion could be related to membrane lipids more than to extracellular lipids. From other investigations, it is still uncertain whether lipids in the forming enamel are true extracellular matrix components or remnants of cell membrane entombed during enamel formation (Goldberg and Escaig 1984c). Although only a small part of the lipids play a role in the enamel matrix, experimental models such as zinc deficiency or essential fatty acid deficiency induce structural alterations in the forming enamel (Goldberg *et al.*, 1990). Some defects were due to the influence of cell shape on the forming enamel. However, we may also conclude from these observations that some minor lipid components may play a role in enamel formation and impair the initial mineralization.

The organic/inorganic relationship in forming enamel still constitutes a controversial question. Enamel proteins just secreted near Tome's processes associate by end-to-end self-assembly. This promotes the formation of bundles of fiber-like structures. These fibrous structures, 30-35 nm in width, stain as glycoproteins. This sub-structure gives a periodicity to the fiber, the forming enamel appearing as a ladder-like structures after fixation, even after rapid-freezing and freeze-substitution fixation (Goldberg and Septier, 1986) (Figs. 14-17). Proteoglycans are likely responsible for the lengthening processes. On sections obtained after rapid-freezing followed by freeze substitution, electron-dense crystal-like structures10 nm in width appear as the first mineralized structures inside these fibers. What is observed is therefore either the edge of developing crystals larger than what is observed, or the early stages of mineralization seen inside ribbon-like structures. The mineral phase was identified as nanometer-sized particles of hydroxyapatite which display many structural defects such as ionic substitutions and deficiencies, screw dislocations, low and high angle boundaries, the latter resulting from the fusion of ribbon-like crystals. (Höhling *et al.*, 1982; Cuisinier *et al.*, 1992). The concept developed by Travis and Glimcher (1964) and Glimcher (1979) fits well with this appearance. Phosphorylated proteins organized in the form of tubules first allow bonding of inorganic calcium followed by accumulation of phosphate inside these enamelin structures. Amelogenins are supposed to be located in the spaces between the tubules. Amorphous calcium phosphate, unstable from a thermodynamic point of view, is at the origin of a cascade leading first to octacalcium phosphate (OCP), and giving rise ultimately to stable HA. After the formation of an initial seed, development by two-dimensional growth in width and in length leads to three dimensional growth (Brown, 1965; Weiss *et al.*, 1981).

As an alternative to this concept, it has also been hypothesized that the continuity between dentine and enamel allows crystals initiated in dentine to grow inside enamel tubule-like structures (Simmons, 1979). This concept denies any nucleating properties to

enamel proteins, which act solely as a guide or influence, by streaming effects or as a thixotropic gel, on the shape of self-aggregating, growing crystals. This matter is still open for discussion.

Cementum

The periodontal ligament is constituted by a mixed cell population: fibroblasts, osteoblasts and cementoblasts. These latter are responsible for the synthesis and deposition of cementum matrix proteins. The first cementum layer deposited by these cells is acellular. At later stages of formation, in the central and apical parts of the roots, the cementum is cellular. Matrix components comprise:

- first, a group of collagen fibers. Type I collagen accounts for more than 90-95%, while type III collagen constitutes between 5-7% (Birkedal-Hansen *et al.*, 1977). Some of these collagen fibers are produced by fibroblasts and incorporated during cementum formation as cementum extrinsic fibers. Others are produced by cementoblasts and become cementum intrinsic fibers.
- second, non-collagenic proteins, including five phosphoprotein fractions very similar to bone and distinct from those of dentine. These proteins are glycosylated sialoproteins. They have the RDG adhesive triplet and have been identified as osteopontin (Glimcher and Lefteriou, 1989). Immunocytochemical studies show that the acellular cementum stains strongly for osteopontin, but not for osteocalcine or dentine sialoprotein. In contrast, cellular cementum stains strongly for osteopontin and osteocalcin, but not for dentine sialoprotein. Cementoblasts and cementocytes also stain for these two proteins. Therefore, the cells express an osteoblast-like phenotype more than an odontoblast-like phenotype (Bronckers *et al.*, 1994).
- third: GAGs from human cementum have been identified as hyaluronic acid (16%), chondroitin sulfate (53-69%) and dermatan sulfate (31%) (Barthold *et al.*, 1988). They are associated with proteins, forming a population of three distinct proteoglycans.

With regard to its composition and mode of mineralization, cementum is similar to bone and dentine. However, it is the softest of all mineralized tissue.

In vitro studies and future studies on biomineralizations

During the last decades, a number of molecules have been identified in mineralized tissues, including dental tissues and structures. They were all evaluated as potential promoters or inhibitors of biomineralization. Initially, collagen was believed to be involved in such processes, mostly because the molecular periodicity along organic surfaces may induce oriented nucleation in inorganic crystallization. However, the concept of epitaxial formation connected with collagen banding and mineralization in the holes or gaps of the quarter staggered structure appears less attractive at present. Thereafter, phosphorylated molecules, including phosphoproteins and phospholipids, became major candidates as mineralization promoters in connective tissues, whereas in enamel a very simplified concept was that enamelins, the mineral-associated phosphorylated proteins, were responsible for such processes. These are the current ideas that predominate nowadays.

In fact, phosphorylated molecules can be visualized at sites where mineralization occurs, i.e. at the mineralization front, either at the junction between predentine and dentine, or between bone and osteoid. In this context, alkaline phosphatase, also present at sites which mineralize, removes organic phosphate from phosphorylated proteins providing phosphate for mineral deposition (Beertsen and Van Den Bos, 1989). However, *in vitro* studies have shown that this occurs only when phosphorylated proteins are adsorbed onto solid supports, and at low concentrations. They may act in some conditions also as inhibitors of nucleation or/and crystal growth (Linde *et al.*, 1989). Many of the phosphorylated proteins so far identified in dental tissues are implicated in cell adhesion and spreading, but their role in mineralization is still unsolved. It must be pointed out that the fact that a protein is located in a mineralized tissue does not necessarily mean that it is a promoting factor for mineralization. On the contrary, its role in mineralized tissue may be that it acts like crystal growth limiting factors.

Along this line of evidence, phosphatidyl serine promotes *in vitro* mineralization in systems in which calcium-PS-phosphate complexes are allowed to form, but inhibits mineralization when incorporated into liposomes. When the surface of hydroxyapatite seed crystals are coated with PS, their rate of proliferation in a metastable solution is reduced (Boskey and Dick, 1991). In this context, it should be reminded that phospholipids are the only material isolated from mineralizing tissues that have been shown capable of causing *in vivo* mineralization in the absence of cells (Raggio *et al.*, 1986).

Other forms of lipids have also been implicated in biomineralization. They take origin from cell debris or are extracellular structures. Matrix vesicles have been identified in cartilage, forming bone and early dentine. These membrane bound structures are the locus of initial crystal formation in some tissues. They are associated with PGs and are alkaline phosphatase-rich (reviewed by Bonucci, 1987). They are enriched in metalloproteinases that degrade proteoglycans (Dean *et al.*, 1992). Crystal ghosts seen after demineralization contain acidic glycosylated and sulfated proteins associated with phospholipids. They are observed in bone, dentine, cartilage and enamel (Bonucci *et al.*, 1988; Goldberg *et al.*, 1995). These two structures, containing acidic phospholipids, would increase Ca^{2+} uptake, followed by dissolution and ion concentration. Loss of water would tend to concentrate Ca^{2+} and HPO_4^{2-}, which provide an environment in which mineralization is facilitated (Vogel and Boyan-Salyers, 1976).

Many molecules may be candidates for promoting nucleation and crystal growth. However, the situation is confusing. Clearly, the same molecules, depending on concentration or how they have been prepared (free or adsorbed), may act as crystal promoters or inhibitors. It is also known now that interactions occur between molecules, for example between proteoglycans and phospholipids (Goldberg *et al.*, 1995), between osteopontin and osteocalcin (Ritter *et al.*, 1992), and more than likely between many other molecules. The formation of such complexes changes the individual properties and consequently the functions of each molecule as studied in an isolated form.

Structural properties of non-collagenous matrix proteins are also confusing. ß-pleated sheets conformations have been detected in dentine phosphoprotein. They can either promote or inhibit hydroxyapatite formation, depending on concentration. It was also reported that amelogenins, which are phosphorylated and hydrophobic proteins, are space-filling molecules which ag-

gregated in a gel phase. Their secondary structure is rich in ß-pleated sheets and ß-turn, resulting in a coiled configuration devoid of α-helices. A component peptide is likely the site of interaction with Ca^{2+}. It forms a spiral through which calcium ions may be transported (Renugopalakrishnan *et al.*, 1989). On the other hand, ß-sheets were not identified by Jodaikin *et al.* (1987) in amelogenins but only in enamelins. The situation became even more confusing when it was recently suggested that most of the non-amelogenin proteins, including enamelins, are serum-derived proteins and not true enamel proteins. Since albumin and other serum proteins can not be candidates for promoting mineral nucleation, and because the first steps of mineralization occur at the dentine-enamel junction on preexisting dentine mineral or mediated by the tuftelin concentrated at the DEJ, amelogenins will serve mostly to control the growth of the mineral phase (Fincham *et al.*, 1992). This stresses the importance of molecules in an amorphous phase, a class of space-filling molecules. In predentine, amorphous proteoglycans and phospholipids fill the empty spaces between the collagen fibers. Analysis of the calcium distribution in predentine by EELS reveals that a significant increase in calcium concentration is observed from the center of the predentine to the distal part next to the mineralization front (Plate *et al.*, 1992). This is an example of the role that amphiphilic molecules, implicated in the concentration of ions before precipitation of initial crystal seeds, may play in mineralizing tissues. Another example is the amelogenins. In both cases, it is not the rod-like protein (collagen fibers and/or enamelin crystal ghosts) which constitute a template and provide an adequate substrate for initial mineralization. At the very best, these structural molecules provide a skeleton which allows calcium and phosphate oriented deposition. Stereochemical preferential interactions have been evidenced between acidic proteins (BSP, dentine phosphoproteins), acting as a template for oriented crystal nucleation, and crystal (OCP and/or HA) growth (Addadi *et al.*, 1992; Veis, 1993). The possibility that organic molecules concentrate and even preorganize the spatial distribution of ions before their organized precipitation has now to be considered.

Along this line of evidence, we have studied in our laboratory *in vitro* mineralization by diffusion in a collagen gel. An experiment was carried out in a type I collagen gel (Boy, Reims, France) diffusion system into which Ca^{2+} was allowed to diffuse from the top of the gel. After a few days of gelification in the presence of phosphate, the network of collagen fibers was crystal-free, in the absence of calcium solution (Fig. 18). In presence of calcium chloride, mineral deposition occurred at the interface gel/solution. SEM observation evidenced flower-like structures seen as petal-like projections (Fig. 19), and identified by infrared analysis and electron diffraction as HA and/or OCP. Using anhydrous methods of fixation and dehydration, TEM allowed visualization of nodule-like structures formed by thin star-like crystallites (Fig. 20). These mineralizations were always located in the spaces between the collagen fibers. When C4-S (70%) and C6-S (30%) (Sigma) were added to the collagen during gelification, alignments of crystal plates occurred along the collagen fibers, which have the appearance of garlands, and only a few crystals were seen in intercollagenic spaces (Fig. 21). The flat crystallites, 1100-1500 nm large and 140 nm thick, displayed an apparent periodicity along the collagen fibers, although calculations showed that variations occurred between 420 -560 nm. The c-axis of the crystals seems to be at right angles to the collagen fibers.

This work is still in progress. At the present stage, two complementary hypothesis may be put forward. It is likely that the coating properties of GAGs change the interaction of collagen with calcium and phosphate ions, and therefore the loci of mineralization. It is also probable that the steric calcium exclusion of GAGs contributes to the precipitation of ions on collagen fibers. GAGs, as amphiphilic molecules, occupy most of the space available and consequently contribute to reduce the areas where calcium is in a free form. Thus, ion concentration may favor initial crystal formation on collagen fibrils.

The question is therefore to determine the role of the two groups of molecules implicated in biomineralization. Either the major role is played by structural molecules, e.g. collagen, enamelins, and/or membrane complexes, providing loci actively taking part in the anhydrous concentration of ions which will further condense and form amorphous calcium phosphate, then eventually OCP and HA; or the major role is ascribed to space-filling amorphous and amphiphilic molecules which may preorganize ion concentration and put their fingerprints to an oriented deposition of calcium phosphate on structures serving mostly as passive support. The two hypothesis may be combined. We believe that this will be one of the major questions under investigation in the future.

Summary

Extracellular matrix components and cell-derived microstructures are implicated in mineralization processes which occur in dental tissues. The respective role(s) of collagenic and non-collagenic matrix components are reviewed: phosphorylated and non-phosphorylated proteins, proteoglycans and phosphpholipids. Space-filling amphiphilic molecules seem to play an important role in the preorganization and oriented deposition of calcium phosphate on structures serving more or less as passive support in dentine as well as in enamel.

KEY WORDS: *dentine, enamel, cell-derived microstructures, biomineralizations, extracellular matrix.*

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