RAT MOLAR TOOTH GERM DEVELOPMENT:
COLLAGEN ELABORATION AND ALKALINE PHOSPHATASE ACTIVITY
DURING DENTINOGENESIS

by

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INTRODUCTION

Developmental Aspects

The tooth develops as a result of the synthetic activity of two specific organ systems; the enamel organ and the dental papilla. The former is an ectodermal derivative from the oral epithelium, while the latter is a mesodermal derivative from the first branchial arch mesenchyme. Evidence indicates that interaction between the two derivatives during tooth development is in the nature of an inductive mechanism (39, 59, 95). The role alkaline phosphatase may play in such a process has not been determined.

The cells of the oral epithelium along the alveolar ridge of both the maxilla and mandible invaginate into the underlying mesenchyme and displace it. The invaginating tissue, the dental lamina, eventually differentiates into the enamel organ and participates in amelogenesis. There is histochemical evidence of alkaline phosphatase activity in the enamel organ (120). Corresponding electron cytochemical evidence indicates that enzyme activity is associated with the plasma membrane of the enamel organ cells (60,66, 67, 120, 212).

The alveolar mesenchyme, displaced and condensed by the invaginating enamel organ, differentiates into the dental papilla and participates in dentinogenesis. The mature dental papilla is characterized by polarized odontoblasts which are organized into an epithelial-like layer that lines the predentin matrix. The odontoblasts are responsible for the elaboration of the predentin matrix, which consists primarily of collagen fibers and ground substance (113). Following the maturation of the predentin matrix, it calcifies to form dentin. Histochem-

ical evidence indicated that alkaline phosphatase activity is present in the dental papilla during dentinogenesis (99).

Alkaline Phosphatase

a. General Considerations

Alkaline phosphatase (E.C. 3.1.3.1.) is ubiquitous in nature; however, its biological role has not been determined even though it has been the subject of a great number of scientific investigations (32). The enzyme is a phosphomonoesterase with optimum activity in the alkaline pH range (8). Alkaline phosphatase is a known element of blood serum chemistry, and its detection and association with known disease states is of clinical significance (55, 56). In the developing tooth, alkaline phosphatase has been shown to be localized in the enamel organ (85), and in the dental papilla (99).

b. Histochemistry

Alkaline phosphatase activity has been demonstrated *in situ* in a wide variety of tissues since the inception of a specific histochemical technique (44, 100). Subsequently, two techniques using different principles for the demonstration of enzyme activity have been emphasized in the literature. First, the modified Gomori technique depends upon the release of free phosphate ions and their subsequent capture by lead (82). Second, the simultaneous coupling technique, originally demonstrated by Menten, Junge and Green (77), involves the cleavage of the phosphate from the substrate and coupling of the substrate with an azo dye (82). Tissue demonstrations of enzyme activity have generally been similar, even though the latter method does not utilize the release of phosphate ions in the actual demonstration. The tooth germ was an early and frequent subject of alkaline phosphatase histochemistry (20, 45, 65, 80, 98, 104,

105, 118, 120).

Alkaline phosphatase activity was demonstrated histochemically in the enamel organ and the dental papilla, although its cellular localization was unknown. During dentinogenesis, enzyme activity was frequently associated with the mature odontoblasts (11, 20, 57, 104, 105, 118). However, numerous investigators could find no evidence of enzyme activity associated with the odontoblasts (45, 90, 103). The controversy of the histochemical demonstration of alkaline phosphatase activity associated with the odontoblasts during dentinogenesis has not been fully resolved.

Enzyme activity has consistently been demonstrated in the subodontoblast layer (20). A capillary plexus is known to be located in the subodontoblast region (91). However, the role of the subodontoblast cells is not known, and the evidence for alkaline phosphatase activity in the subodontoblast cell region has clarified neither the cells' biologic activities nor their relationship to dentinogenesis.

Differences in tissue preparation, including fixation and embedding, may be factors that have contributed to the inconsistencies in the demonstration of the enzyme. Both fixed and non-fixed tissues have yielded similar and dissimilar results with regard to the distribution of the enzyme reaction product. Many of the early demonstrations of the enzyme may be incorrect because paraffin embedding requires exposure to temperatures of 55 to 60°C and this heat range has subsequently been shown to be inhibitory to alkaline phosphatase activity (46). Because of tissue preparation differences and resulting discrepancies in histochemical demonstrations of alkaline phosphatase, the previous demonstra-

tions of enzyme activity in the developing tooth need critical evaluation.

c. Electron Cytochemistry

Two major modifications of the original Gomori (44) lead salt technique have been extended for the demonstration of alkaline phosphatase activity at the electron microscope level. Hugon and Borgers (51) using lead nitrate as a capture reagent devised a multistep procedure designed to establish phosphatase activity at pH 9.0. A one step incubation procedure was developed using lead citrate as a capture reagent at a pH of 9.4 (76). Both techniques have been used to demonstrate alkaline phosphatase activity in the tooth germ.

Yoshiki and Kurahaski (120), using a lead citrate procedure (76), demonstrated that alkaline phosphatase activity is dispersed in the mantle predentin matrix and is associated with the preodontoblast membrane. However, incubation times were not given. Using the Hugon and Borgers (51) cytochemical technique, Fromme et al., (37) demonstrated membrane associated enzyme activity in the preodontoblasts. Leonard and Provenza (65, 66) using a variety of electron cytochemical techniques, described enzyme activity in the mantle predentin matrix and along the lateral membrane of the mature odontoblasts. Finally, Larsson (61), while studying matrix vesicles, identified alkaline phosphatase activity in the mantle predentin matrix in addition to enzyme activity associated with the matrix vesicles. In each case, the results of the enzyme distribution demonstrated in the predentin matrix were limited to a single developmental stage. Thus, a relationship between alkaline phosphatase activity and either matrix differentiation or odontoblast differentiation

has not been established.

The electron microscopic cytochemical findings have generally reflected the tissue distribution of alkaline phosphatase activity found by histochemical means. Fixation of the tooth germ varied from study to study as did the buffering system; however, these procedural differences did not appear to create dramatic differences in the distribution of reaction product in the dental papilla. The length of the incubation period is an important factor in the distribution of reaction product (82), but the time element was not clearly described in any of the afore mentioned studies. There has been little published evidence to indicate how incubation time relates to enzyme distribution in the tooth germ. Generally, however, the longer the incubation period, the greater the likelihood of diffusion of the enzyme and reaction product from the *in situ* location (82).

d. Biochemical Activity

Biochemical studies of alkaline phosphatase from dissected odontoblasts have been performed (69). The optimum activity of alkaline phosphatase for the whole tooth germ was at pH 10.3 (119). Optimum enzyme activity occured at pH 10.1, for enzyme extracted from either the isolated enamel organ (35, 36) or the isolated dental papilla (46). The majority of histochemical and electron cytochemical studies were conducted at a pH of 8.7 to 9.5, but the relationship between the pH of optimum enzyme activity and the biological role of the enzyme has not been determined. Also, evidence is lacking which specifically relates enzyme distribution as a function of pH.

e. Fiber Formation

Alkaline phosphatase was hypothesized to play a role in collagen biosynthesis based on observations of wound healing experiments (25, 31). Histochemical evidence indicated high alkaline phosphatase activity associated with regenerating collagen fibers. Bradfield (17, 18) demonstrated that alkaline phosphatase activity was associated with fibrous protein secretion. The biochemical results of Gold and Gould (41) indicated an apparently strong affinity of collagen for alkaline phosphatase. Thus, the evidence suggests a relationship between alkaline phosphatase activity and collagen fibrillogenesis.

f. Calcification

Alkaline phosphatase activity was associated with calcification as initially promulgated for bone by Robison (87). This hypothesis was based on the assumption that the enzyme acted to liberate phosphate ions for subsequent precipitation into hydroxylapatite crystals. The relationship between phosphatase activity and calcification has been reviewed by Bourne (15). Both inorganic pyrophosphatase and ATPase (26, 27, 29, 68, 112) have been identified as specific enzyme components of nonspecific alkaline phosphatase extracted from developing bone. Pyrophosphatase activity has been described in the alkaline phosphatase activity of the developing tooth germ (119).

Since it has been shown that pyrophosphate inhibits calcification (30, 33), enzymatic cleavage of the pyrophosphate by alkaline phosphatase would presumably enhance the calcification process. Not only would an inhibitory factor be diminished by alkaline phosphatase activity, but the resulting additional phosphate groups would favor calcification.

Matrix vesicles found both in developing bone matrix (5, 12) and in the mantle predentin matrix (9, 28, 61, 94, 96) have been shown to possess alkaline phosphatase activity, pyrophosphatase activity and ATPase activity (61, 73). It has been hypothesized that the vesicles play an integral role in calcification of bone and dentin (1, 6, 19, 30, 53). The matrix vesicle may act as a nucleation center for the initiation of calcification. The matrix vesicles have been found in the mantle predentin matrix, but not in the circumpulpal predentin matrix (9, 94). Thus, if matrix vesicles are an integral part of mantle dentin calcification, then an alternative mechanism must exist which initiates and controls calcification of circumpulpal dentin.

g. Dentinogenesis

Even though the general location of alkaline phosphatase activity in the dental papilla during dentinogenesis is known, no study has identified the distribution of alkaline phosphatase activity during sequential development of odontoblasts or predentin matrix. An understanding of the possible functional role of alkaline phosphatase in dentinogenesis can best be obtained by careful analysis of the relationship between the presence of enzyme activity and the events of odontoblast and matrix differentiation. The ultrastructural relationship between the elaboration of the predentin matrix collagen by the odontoblasts and alkaline phosphatase activity has not been described.

Collagen Biosynthesis in Dentinogenesis

There is a great deal of controversy concerning the intracellular transport of collagen and the means by which the molecule exits from the odontoblast. Two major theories have been promulgated concerning

the biosynthesis, transport, and extrusion of collagen by the odontoblast into the predentin matrix. The first espouses transcription on the RER, packaging in the Golgi into the secretory "granules", migration of the "granules" to the odontoblast process, and extrusion of the collagen from the "granules" into the matrix via exocytosis (116, 117). The second theory places transcription at the RER, but the migration and the extrusion of the collagen molecule does not involve secretory "granules". Instead, the extrusion process involves movement of the collagen molecule from the cytosol, without necessarily passing into the Golgi complex for packaging, and the passage of the molecule through the membrane of the odontoblast into the predentin matrix (54, 84).

Statement of the Problem

The transition from intracellular procollagen to extracellular tropocollagen has eluded a precise and predictable description by electron microscopy. Even though intra- and extracellular appearance has been described, the ultrastructure of the extrusion of collagen from the odontoblast is poorly understood and the cellular mechanism involved has not been determined. In order to clarify the extrusion process, it is necessary to describe continuity between the intracellular procollagen and the extracellular topocollagen, which is identifiable as the banded collagen fiber. Since the apical end of the odontoblast is the known predictable site of matrix collagen extrusion, it provides the transition point at which the ultrastructural features of collagen extrusion can be examined.

The purpose of this study is to examine two aspects of dentinogenesis: (1) the maturation of the odontoblasts and of the predentin

matrix, and (2) the extrusion of collagen into the predentin matrix by the mature, secretory odontoblast. Studies on the maturation of the matrix will focus on the sequential differentiation of the odontoblasts and the association of matrix vesicles to the initiation of calcification of mantle dentin. The ultrastructural features of collagen extrusion will be studied in the mature secreting odontoblast. Continuity of collagen production from an intracellular position to an extracellular position will be established at the ultrastructural level. Alkaline phosphatase activity will be related histochemically and electron cytochemically to the development of the predentin collagen by the odontoblast. The presence of enzyme reaction product will be correlated to functional processes at different stages of dentinogenesis. It will be determined if the presence of this enzyme can act as a reliable indicator of a particular stage of tooth development.

MATERIALS AND METHODS

Materials

Albino, Sprague-Dawley rats were bred and the offspring were used to provide first molar tooth germs. Neonates and 2 day old pups were used. The tooth germs were dissected from the dental arch with the aid of a dissecting microscope and placed in Tissue Culture Medium 199 (DIFCO) until the tissue was processed. This period did not exceed ten minutes. Molars from twenty pups from five litters were used.

Methods

a. Histochemistry for Light Microscopy

The tooth germs were quickly frozen in liquid nitrogen and stored in air-tight containers at -27° C, until sectioned at 7 microns in a Tissue Tek Cryostat. The sections were transferred serially onto cover slips and allowed to dry at room temperature. Both lead salt (44) and the azo-dye simultaneous coupling techniques (82) were used to demonstrate alkaline phosphatase activity in the non-fixed frozen sections.

A modification of the Gomori (44) lead salt technique was used. Sections were incubated for 5, 10, and 20 minute periods at a pH of 9.5 in a medium containing 0.20 M sodium beta glycerol phosphate (Sigma), 0.16 M sodium diethyl barbiturate buffer (Merck), and 0.015 M magnesium chloride. Following incubation, the sections were immersed in 1% calcium chloride for 2 minutes, then in 2% cobalt nitrate for 5 minutes, rinsed in distilled water and developed in 1% ammonium sulfide for 2 minutes. Sections incubated in the lead salt medium were either dehydrated in ethanol, cleared in xylol and mounted in Permount, or were mounted directly in glycerol gelatin.

The simultaneous coupling technique as modified by Pearse (82) was used. The incubation medium for the azo dye coupling reaction consisted of 0.20 M AS-BI phosphate*(Sigma), 0.10 M Tris-HCl buffer and 1.0 mg/ml azo dye Fast Red TR (Sigma). The incubation was conducted at 37° C with pH 9.5, for periods of 5, 10 and 20 minutes. Sections incubated in either of the above reaction mixtures were counter stained in nuclear fast red, methyl green or Mayer's hematoxylin. All sections reacted in the simultaneous coupling mixture were mounted directly in glycerol gelatin (Sigma).

The histochemical demonstration of alkaline phosphatase activity is based on capturing _in situ_ a molecular component of the substrate following the hydrolytic action of the enzyme. In this study, two different methods were used to capture and demonstrate the components of the hydrolized substrate. First, the modified Gomori technique uses a lead capture reagent to bind the phosphate group hydrolyzed from beta-glycerolphosphate substrate. In this way, enzyme activity is demonstrated by phosphate captured _in situ_.

Second, the simultaneous azo dye coupling technique uses a soluble azo dye to couple with the residue component of the substrate (AS-BI phosphate) following the release of the phosphate group. The demonstration of the enzyme's activity depends on the precipitation of the azo dye with the residue and the identification of the phosphate group is not considered. Thus, by demonstrating both components of the substrate independently, the _in situ_ localization of alkaline phosphatase activity is more clearly defined.

*Phosphate ester of 7-Bromo-3-hydroxy-naphth-o-anisidide.

b. Tissue Preparation for Electron Microscopy

First molar tooth germs from albino, Sprague-Dawley rat pups, both neonates and two day olds, were dissected and immersed in fixative containing 2.0% glutaraldehyde (Polysciences, Inc.), 1.0% paraformaldehyde (Sigma) (prepared fresh), and 0.067 M cacodylate buffer (Sigma) at pH 7.3 and 4.0^{o} C, for 60 minutes. The tooth germs were rinsed twice for 20 minutes in 0.067 M cacodylate buffer (pH 7.3, 4.0^{o} C). The tooth germs were embedded in 5% agar-agar (DIFCO), sliced at 50 mu with a Smith-Farquhar tissue chopper (Sorvall), and the slices were pooled in buffer.

c. Electron Cytochemistry

Specimen slices were incubated in the complete reaction mixture for 10 minutes according to the new lead citrate method (76). The incubating medium consisted of 1.4 ml of 0.2 M tris-HCl buffer, pH 8.5, 2.0 ml of 0.1 M sodium beta-glycerolphosphate, 2.6 ml of 15 mM magnesium sulfate, and 4.0 ml of saturated alkaline lead citrate solution, pH 10.0. Final pH was adjusted to 9.5 with 0.1 N NaOH. An incubation period of 10 minutes was used in order to reduce the potential diffusion artefact which is known to result from longer incubation periods (82).

d. Substrate Free Control

The substrate free control specimens were incubated in the reaction mixture without the substrate, beta-glycerolphosphate. All other conditions such as pH, temperature, and length of incubation period were consistent with the complete reaction mixture incubation. Substrate free control specimens were viewed both unstained and stained with a lead stain. This procedure enabled the nature of the staining artefact which

may result from the lead staining to be determined. Staining the tissue with heavy metal stains such as uranyl acetate and lead citrate prior to viewing tends to enhance the contrast of the tissue and this must be differentiated from the lead deposit as a result of alkaline phosphatase activity. It was determined that the staining artefact from the lead stain was identifiable as compared to nonstained sections and could be excluded wherever necessary.

e. Cloudy Control

Tissue specimens treated with EDTA, an inhibitor of tooth germ alkaline phosphatase activity (69), constituted the cloudy control. Specimens were treated in the usual manner except that 0.10 M sodium phosphate dibasic buffer was substituted for the magnesium sulfate in the reaction mixture. The phosphate of buffer origin presumably precipitated with the lead of the lead citrate solution and formed lead phosphate. The precipitate clouds the reaction mixture, hence the name. This control procedure aids in determining if any tissue component has an affinity for lead phosphate without any intervention of alkaline phosphatase activity. If a generalized deposit of lead phosphate were observed, it could be stated that the tissue components have an equal but undetermined affinity for the precipitate.

f. Non-fixed Control

Correlation of the histochemical results with the electron cytochemical results provides an important control for the demonstration of alkaline phosphatase activity. The non-fixed frozen sections demonstrated enzyme activity without the effects of fixation. By comparing the distribution of enzyme activity in the fixed electron cytochemical

specimens with the non-fixed histochemical specimens, the possible changes in localization may be estimated. While histochemical sections were 7-9 microns, the cytochemical sections were 80–90 nm thick. Because of this difference in thickness, it would be expected that the intensity of the observable reaction product would be reduced in the electron cytochemical specimens.

g. Light microscopic preparations were photographed with a Zeiss Photoscope III. Electron microscopic preparations were viewed with a Phillip's 301 Electron Microscope using an accelerating voltage of 70Kv.

RESULTS

Developmental Aspects

The dental papilla originates from the branchial arch mesenchyme, and following the developmental changes, the papilla participates in the formation of dentin. Mantle predentin matrix develops in the mesenchyme adjacent to the basal lamina of the enamel organ. In the first molar of the neonate rat, the least differentiated region of the mantle predentin matrix is at the point of initial interaction between the undifferentiated mesenchyme and the leading edge of the enamel organ. The cusp represents a region of more advanced development than the leading edge region because it is where mesenchymal-epithelial interaction first occurred. Hence, the mantle predentin matrix between the leading edge of the enamel organ and the cusp region represents a developmental continuum.

The neonate (first molar) tooth germ in figure 1 exemplifies this continuum as various stages of matrix differentiation can be identified. Point A represents the matrix in the undifferentiated mesenchyme, at the stage of initial interaction with the leading edge of the enamel organ. Point B is the cusp region or that of greatest differentiation. Therefore, the region between points A and B represents different degrees of mantle predentin matrix differentiation. In the neonate there is no evidence of enamel or dentin at the cusp tip of the first molar. This state of development is in contrast to the cusp region following two days of development (figure 2). By two days of development, enamel and dentin are evident and the polarized odontoblasts have matured. The predentin matrix, shown sandwiched between the calcified dentin and the odontoblasts, is elaborated by the secretory odontoblasts.

Observations in the current study demonstrate specific sequential stages of mantle predentin matrix differentiation (figure 1). First, the initial stage of mantle predentin matrix is in the mesenchyme adjacent to the leading edge of the enamel organ (point A). Second, the immature stage of mantle matrix is adjacent to the inner enamel epithelium of the enamel organ (between points A and B). Third, the mature stage of mantle predentin matrix is in the cusp region of the neonate molar and represents a more advanced stage just prior to calcification. Fourth, the mantle dentin calcification stage of mantle predentin matrix is accompanied by the formation of the circumpulpal predentin matrix (figure 2). This stage is characterized by calcified dentin and enamel and it occurs in the molar after two days of postnatal development.

Initial Stage

a. Histochemistry

The earliest predentin matrix, comprised of undifferentiated mesenchyme, is shown in figure 3 at the point of initial interaction with the leading edge of the enamel organ (this represents point A in figure 1). Alkaline phosphatase activity does not appear as a prominent feature of this early stage of differentiation of the matrix. Reaction product is not evident in the leading edge, but it is prominent as development proceeds in the enamel organ. The undifferentiated mesenchyme exhibits a generalized but light deposition of reaction product.

b. Morphology

The interaction between the mesenchyme and the adjacent leading edge of the enamel organ is shown at higher magnification in figure 4. The basal lamina of the enamel organ is discrete and forms a continuous

barrier separating the cells of the enamel organ and the elements of the mesenchyme. The close, proximal association of the two tissues results from the invagination of the mesenchyme by the enamel organ. However, the nature of this interaction is not fully understood. The mesenchyme typically contains cells with attenuated cytoplasmic processes and the paucity of collagen fibers is typical.

A portion of this initial predentin matrix is shown at higher magnification in figure 5. The basal lamina of the enamel organ features fine collagen fibrils which penetrate into the mesenchyme. The lack of a distinct, banded fiber structure is generally typical of basal lamina collagen of epithelial origin. The mesenchyme cell lies in close approximation to the basal lamina and appears to be contributing collagen to the extracellular framework of matrix fibers. The mesenchyme cell represents the earliest preodontoblast, and a close proximal relation has been established between the mesenchyme cell and the enamel organ. Collagen fibers from both tissues contribute to this early predentin matrix and the sparse collagen fiber density is evident.

A column of fibers in longitudinal section appears to be extruding directly from the base of the cell process into the presumptive predentin matrix space. An adjacent group of fibers are seen in cross section next to the mesenchymal cell and may represent the same group. The mesenchyme cells, the very early preodontoblasts, appear capable of extruding a uniform group of collagen fibers from near the base of a cell process into the initial mantle predentin matrix.

c. Cytochemistry

Alkaline phosphatase activity was not a feature of the predentin

matrix at its initiation as evidenced by the lack of reaction product (figures 4 and 5). This evidence correlates with the histochemical results shown in figure 3.

Immature Stage

a. Histochemistry

The light micrograph in figure 6 illustrates a histochemical preparation of the enamel organ and the adjacent mesenchyme of the dental papilla. At this stage of differentiation, the mantle predentin matrix is immature. This region is located about midway between points A and B (figure 1). By comparison to the situation in the initial predentin matrix (figure 3), the mesenchymal cells of the immature predentin matrix appear in greater density along the basal lamina of the enamel organ. Delineation of the differentiating mantle predentin matrix space is evident. Alkaline phosphatase activity appears dispersed in the mesenchyme of the dental papilla, but appears most noticeably in the region of increased cell density parallel with the predentin space. In the enamel organ, the red reaction product is also evident in the stratum intermedium cell layer but not in the inner enamel epithelium.

b. Morphology

A region of the immature mantle predentin matrix is shown in the electron micrograph in figure 7. This electron micrograph shows an overview of the immature matrix and alignment of the preodontoblasts. Fibers emanate from the continuous basal lamina into the matrix space and cell processes from the preodontoblasts also extend into the space. Initial signs of polarization of the preodontoblasts are evident as the major organelles appear between the basal nuclear region and the apical region

adjacent to the predentin matrix space. The intercellular space between the neighboring preodontoblasts is greatly reduced when compared to the space between neighboring mesenchymal cells which were found at the initial predentin matrix (figure 4). This reduction eventually leads to a sequestering of the matrix between the preodontoblasts and the basal lamina. Junctional complexes, a characteristic of epithelial cell layers and not generally a prominent feature of mesenchyme, can be seen between adjacent preodontoblasts. The narrowing of the space between the preodontoblasts and the development of intercellular junctions diminishes the potential contribution of fibers from the subodontoblasts to the predentin matrix.

Mantle predentin matrix at the immature stage of development is illustrated in figure 10. Banded collagen fibers are evident in the matrix as are fibers emanating from the basal lamina. The differentiating inner enamel epithelial cells adjacent to the mantle predentin matrix exhibit numerous mitochondria between the cell nucleus and the basal lamina surface of the cell. Within the mitochondria, electron opaque granules are apparent.

A region of the predentin matrix at the immature stage of differentiation similar to figure 7 is shown in figure 8. The density of the collagen fibers of the immature predentin matrix was greater when compared with the paucity of fibers in the initial predentin matrix (figure 5). Furthermore, the fibers appear randomly oriented compared to the ordered groups of fibers noted in figure 5. Extracellular matrix vesicles, not previously observed in the initial mantle predentin matrix, are located within the extracellular space of the mantle predentin matrix.

The fine structure of the extracellular vesicles in the immature predentin matrix are shown in figure 9. In this high resolution electron micrograph, the vesicle membrane is distinct. It is trilaminar in appearance with some localized regions of irregularities and elongated, crystalline-like structures are evident within the vesicles.

c. Cytochemistry

Light amounts of alkaline phosphatase reaction product are dispersed evenly in the extracellular matrix space and localized on the matrix vesicles (figures 8 and 9). Mantle predentin matrix also at the immature stage is illustrated in figure 10. The distribution of reaction product appears associated with the fibrillar elements of the predentin matrix and the basal lamina. A single identifiable matrix vesicle appears devoid of electron opaque reaction product.

Mature Stage

a. Histochemistry

The histochemical distribution of enzyme reaction product within the tooth germ at the mature stage of mantle predentin matrix development was similar to that of the immature stage (figure 6).

b. Morphology

A region of mature mantle predentin matrix (point B, figure 1) is shown in the electron micrograph in figure 11. The basal lamina of the enamel organ forms a continuous and distinct boundary of the mature mantle predentin matrix. In this nearly longitudinal section, the preodontoblasts are polarized with a basal nucleus and apical cell processes which extend into the predentin matrix space. As the preodontoblasts mature, they align into a columnar epithelial-like layer. The inter-

cellular space between neighboring preodontoblasts has been greatly reduced, in comparison with the intercellular space which was observed in the earlier stages of development (figures 3 and 7). Junctions are a prominent feature at the apical end of the odontoblast lateral membrane. The close epithelial alignment contributes to the isolation of the mature matrix. This provides the preodontoblasts with the primary contributory role in the formation of the predentin matrix and the alignment diminishes possible contribution to the predentin matrix by the remaining cells of the dental papilla.

A portion of mature predentin matrix which extends from the basal lamina to the apical cytoplasm of the preodontoblasts is shown at high magnification in figure 12. In cross section the basal lamina appears continuous and regular (figure 11), whereas in tangential section it appears continuous but irregular (figures 12 and 13). The collagen fibers appear noticeably more dense in the mature mantle predentin matrix (figures 12 and 13) than in the immature matrix (figure 8). In figure 12, the odontoblast processes project into the dense collagen framework and extend to the basal lamina (figure 12). Junctional complexes between adjacent preodontoblasts are prominent membrane features. Matrix vesicles are electron opaque in the mature predentin matrix and are stationed near the basal lamina of the enamel organ. This feature of the mature matrix (figures 12 and 13) is contrasted with the uniform distribution of the matrix vesicles in the immature predentin matrix (figure 8). The density of the electron opaque material associated with the vesicles appears to increase as the differentiating mantle predentin matrix matures.

A region of the mature mantle predentin matrix, similar to the one

outlined in the previous figure, is shown in figure 13. The basal lamina appears highly irregular in this tangential view. Preameloblast cell processes extend into the predentin matrix space. Collagen fibers, with characteristic banding patterns, appear dispersed throughout the matrix space and even penetrate the basal lamina. Cell processes of odontoblasts, which appear in both the initial and immature predentin matrices, are also prominent in the mature mantle predentin matrix.

c. Cytochemistry

Alkaline phosphatase reaction product, besides being localized in the matrix vesicles, is dispersed nonspecifically throughout the extent of the matrix.

Mantle Dentin Calcification

a. Histochemistry

Following maturation of the organic predentin matrix, it calcifies to form dentin. This process is accompanied by the cellular differentiation of preodontoblasts into mature secreting odontoblasts. Mantle dentin is evident in the cusp region of a molar from a two day old postnatal rat. It is sandwiched between enamel and the circumpulpal predentin matrix (figure 14). This figure demonstrates the presence of concentrated reaction product in the stratum intermedium and stellate reticulum of the enamel organ and, in contrast, the absence of reaction product in the ameloblast cell layer is evident. Alkaline phosphatase activity also is evident along the interface between the apical end of the odontoblasts and the predentin matrix.

b. Morphology

The electron micrograph in figure 15 depicts the initial dentin formation in the cusp region of the first molar of the two day rat. Mantle

dentin, classically described as the first dentin formed in the predentin matrix space, lies adjacent to the basal lamina of the enamel organ. Two continuous ribbons of calcification, A and B, (figure 15) delineates the extent, or width, of the mantle dentin. While mantle dentin comprises the peripheral, initial dentin, circumpulpal dentin comprises the majority of the dentin, and begins to form from the edge (ribbon B) of the mantle dentin. Circumpulpal dentin is produced by the mature, polarized odontoblasts, and this synthetic activity requires a shift of the cell position toward the pulp and away from the apical site of matrix elaboration. The polarized odontoblasts, tangentially sectioned in figure 15, appear as a close, columnar epithelium that lines the circumpulpal predentin matrix.

c. Cytochemistry

Alkaline phosphatase activity is evident in the predentin matrix in a zone adjacent to the apical ends of the mature, secretory odontoblasts. However, reaction product is not evident in the matrix along the calcification front (B). At this stage of development of the mature odontoblasts, enzyme activity is evident along the lateral membrane. In earlier stages of development which occurred prior to initial calcification, enzyme reaction product could not be localized on the lateral membranes of the odontoblasts (figures 11 and 12).

Circumpulpal Predentin Matrix

a. Histochemistry

Alkaline phosphatase activity is evident in the predentin matrix adjacent to the apical end of the odontoblast, i.e., at the odontoblast-predentin matrix interface (figure 14).

b. Morphology

A region of mantle dentin, circumpulpal predentin matrix, and the mature, secreting odontoblasts are shown in figure 16. The collagen remaining in the uncalcified matrix of the mantle dentin appears indistinct as calcification proceeds. Matrix vesicles, once stationed near the basal lamina (figures 8, 12, and 13), are no longer evident in the calcified mantle predentin matrix. In the circumpulpal predentin matrix, a large, organized bundle of collagen fibers (the von Korff fibers) merge directly with the calcification front.

The merging of von Korff fibers with the calcification front is shown at higher magnification in figure 17. The distinctive banded nature of the collagen fibers is enhanced in areas of the von Korff fibers undergoing calcification. The fibers are distinct and typically banded and thus differ from the non-banded fibers noted in the calcifying mantle dentin (figure 16).

The majority of the smaller collagen fibers are oriented in different planes relative to the von Korff fibers and the odontoblast process (figures 16 and 17). These small fibers form a density gradient in the circumpulpal predentin matrix in which the denser and more mature fibers are located adjacent to the calcification front while the less dense and most recently formed fibers are found near the apical end of the odontoblast. The concentration of fibers in the circumpulpal predentin matrix (figure 16) appear greater when compared to the density of fibers observed in the immature and mature mantle predentin matrices (figures 8 and 13, respectively). This increased collagen fiber population appears to correspond to developmental changes in the odontoblasts.

b. Cytochemistry

Once calcification has begun, reaction product appears associated with the lateral odontoblast membrane and the intercellular junctional complexes (figures 15 and 18). Reaction product was associated occasionally with the membrane of the odontoblast process. The deposition of reaction product in the predentin matrix compared to the lateral odontoblast membrane appears to be qualitatively different (figure 18). Along the lateral odontoblast membrane, reaction product forms small crystals of the lead phosphate. These crystals appear associated with the outer leaflet and they protrude into the extracellular space between the adjacent odontoblasts. In the predentin matrix, larger oblong crystals of reaction product are more abundant and are usually associated with collagen fibers.

Control Specimens

a. Substrate Free Control

The apical region of the mature odontoblast and adjacent circumpulpal predentin matrix are shown in the substrate free control specimen in figure 19. Lead deposits were not evident in the matrix or along the lateral mambranes, compared to the pattern of reaction product deposition in figure 18. A region of dentin calcification is evidenced by the crystalline-like structures that are associated with the mature collagen fibers. Although this specimen was stained with both uranyl acetate and lead citrate, staining artefact was not apparent.

b. Cloudy Control

The apical region of an odontoblast, similar to the one in the substrate free control (figure 19), is shown in the cloudy control

specimen in figure 20. A generalized and apparently non-specific deposit of lead phosphate is localized both intra- and extracellularly. This diffuse deposit of precipitate is in contrast with the discrete localization of reaction product in figure 18.

Collagen Elaboration: Odontoblasts and the Predentin Matrix

a. Morphology

The odontoblast layer and the circumpulpal predentin matrix, which is elaborated by the odontoblasts, are illustrated in the molar of a two day old rat in the light micrograph in figure 21. Note the close relationship between the apical ends of the odontoblasts and the predentin matrix. A similar region is shown at higher magnification in figure 22. The predentin matrix is isolated from the capillaries of the underlying subodontoblast region by junctional complexes. These complexes are located consistently at the apical end of the odontoblasts' lateral membrane. The apical cytoplasm of the odontoblast appears nearly devoid of any major organelle system, while the supranuclear cytoplasm exhibits an extensive rough endoplasmic reticulum, large Golgi complexes, and numerous, dispersed mitochondria. The supranuclear cytoplasm comprises the majority of the odontoblast cytoplasm and lies between the apical cytoplasm and the basal nucleus.

b. Cytochemistry

Alkaline phosphatase activity is localized along the lateral odontoblast membrane and in the predentin matrix adjacent to the apical membrane (figure 22). Enzyme activity is also evident in the junctional complexes between odontoblasts.

Intracellular Organization of Collagen

a. Morphology

This section of the results will focus on the organization and extrusion of collagen from cytoplasmic secretion "granules" or packages which are present in the odontoblast during dentinogenesis. The term secretion package is used in place of the term secretion "granule" in order to expand the descriptive term to include procollagen inclusions which appear elongated or irregular (figures 23, 24, 25, 26, and 27). These packages most probably represent the intracellular organization of collagen. They exhibit a variable morphology and usually contain electron opaque particles. They are identifiable in the Golgi region (figure 23), along the lateral plasma membrane (figures 22, 24, and 25) and in the apical cytoplasm (figures 22, 24, 25, 26, and 27). The different shapes of the packages throughout the extent of the cell are a reflection of sectioning the packages in various planes. Secretory packages are frequently elongated (figures 22, 24, 25, 26 and 27) and may appear aligned in rows (figure 24). The packages are characterized by electron opaque particles that are arranged at periodic intervals (figures 23 and 26).

In the Golgi complex, an elongated secretion package is apparent and is identifiable by the electron opaque particles which exhibit a 320 nm periodicity (figure 23). The fibrillar element in the secretory package appears over 1000 nm in length and it consists of several periodic segments. Electron opaque particles are evident in other regions of the Golgi (figure 23). They appear to be associated with an electron opaque material similar to the longitudinal fibrillar element, but without a longitudinal orientation. Adjacent to the Golgi, cisternae of the

rough endoplasmic reticulum contain flocculant material but no electron opaque particles.

Secretory packages, in both longitudinal and cross sections, are located in the apical cytoplasm among an array of microtubules and microfilaments (figure 26). All packages appear membrane limited and contain periodic electron opaque particles. In longitudinal sections, the package contents, procollagen, appear as fine longitudinal fibrils, while the cross sections exhibit a punctate appearance. In either a longitudinal or a cross section of the package, the electron opaque particles appear at the periphery of the package, but always within the limiting membrane (figure 26). The particles shown in the package cross section appear to be arranged at 90° intervals around the perimeter. In longitudinal sections of the package, the particles appear irregular or staggered and most appear 320 nm apart. An elongated, bending secretory package with electron opaque particles is shown in figure 27.

b. Cytochemistry

Alkaline phosphatase reaction product was not associated with the intracellular secretory package. However, it was a consistent feature of the lateral odontoblast membrane (figures 22, 23, 24, 25 and 26).

Extrusion of Collagen: Intracellular Events

a. Morphology

In the supranuclear cytoplasm of the odontoblast, secretory packages are located along the lateral membrane (figures 28, 29, and 30). In this region, vesicles with electron lucent centers begin to appear closely associated with the secretory packages (figure 28). The vesicles appear membrane bound and exhibit a protrusion or membrane extension which

appears to attach to the secretory package membrane (figures 28, 29, 31, and 35).

The vesicle membrane becomes continuous with the secretory package membrane and this gives the appearance of an electron lucent "blister" on the secretory package (figure 30). The fusion of the vesicle with the secretory package results in the confluence of the contents of the vesicles with the procollagen contents of the secretory package. This membrane fusion forms an enlarged limiting membrane of the secretory package.

The electron lucent "blister" appears to expand into a continuous electron lucent space which isolates the secretory package collagen contents from the secretory package limiting membrane (figures 31 and 32). Thus, both the vesicle membrane and the secretory package membrane appear to contribute to the limiting membrane surrounding the electron lucent space. Vesicles may also attach to the limiting membrane when the continuous electron lucent space is present (figures 31 and 35).

b. Cytochemistry

Alkaline phosphatase activity was evident along the lateral odontoblast membrane (figures 28 and 30). However, enzyme activity was not a feature within the odontoblast and, more specifically, reaction product was not associated with the secretory package nor with the electron lucent vesicle.

<u>Intermediate Collagen</u>

a. Morphology

The intermediate form of collagen may be defined as an irregular, fibrous structure without evidence of periodic electron opaque particles and separated from the limiting membrane of the package by a continuous electron lucent space (figures 31 and 32). While the procollagen in the

secretory package was characterized by thin fibrils oriented in a longitudinal fashion (figures 23 and 26), the intermediate collagen was fibrous in nature but without apparent organization (figures 31 and 32). The intermediate form of collagen appears to be the result of the interaction between the vesicle and the secretory procollagen package (figures 28, 31, and 35), with the subsequent loss of the characteristic procollagen features (figures 24, 25, 26, and 27).

b. Cytochemistry

The intracellular, intermediate collagen does not exhibit alkaline phosphatase activity (figures 31 and 32).

Extracellular Intermediate Collagen

a. Morphology

The limiting membrane of the intracellular intermediate collagen (figures 31 and 32) appears to fuse with the lateral membrane of the odontoblast near the apical end of the cell in the region of the intercellular junctions (figures 33, 34, and 35). The limiting membrane of the intermediate collagen is continuous with and is indistinguishable from the lateral membrane of the odontoblast. Thus, the lateral odontoblast membrane now appears as the intermediate collagen limiting membrane.

The fibrous intermediate collagen appears attached to the lateral membrane by fine filaments which bridge the electron lucent space (figures 33 and 34). The intermediate collagen is shown lying between adjacent odontoblasts and is now extracellular (figures 33, 34, and 35). However, it does not appear to be exposed to the predentin matrix.

b. Cytochemistry

Alkaline phosphatase activity can be identified at the periphery

of the intermediate collagen as well as with the limiting membrane, which is now synonymous with the lateral membrane of the odontoblast (figures 33 and 34). The appearance of reaction product associated with the intermediate collagen is in contrast with the apparent absence of reaction product associated with the intracellular intermediate collagen (figures 31 and 32). Once the limiting membrane of the intermediate collagen fuses with the lateral membrane of the odontoblast, alkaline phosphatase activity becomes associated with the limiting membrane (figures 33 and 34). The cloudy control specimen shown in figure 35 does not exhibit alkaline phosphatase activity as noted by the lack of reaction product.

Transport of Collagen: Intracellular to Extracellular

a. Morphology

The transport of collagen appears to be evident in one of the portions of 3 odontoblasts shown in figure 36. In one odontoblast, both intracellular procollagen (secretory package a) and modified intracellular procollagen with an apparent electron lucent space (package b), are identifiable. Extracellular intermediate collagen (ICo or c) is shown between adjacent odontoblasts. The limiting membrane of the intracellular collagen, i.e., packages, a and b, eventually may fuse and become continuous with the lateral membrane of the odontoblasts (package c).

b. Cytochemistry

Alkaline phosphatase activity appears associated with the lateral membranes of the odontoblasts, which includes the limiting membrane of the extracellular intermediate collagen, package c (figure 36). Enzyme activity was not a feature of the intracellular secretory packages a and b.

Intermediate Collagen: Origin of the Banded Collagen Fiber

a. Morphology

The intermediate form of collagen is shown located between adjacent odontoblasts with the apical odontoblast membrane acting as the limiting membrane of the intermediate collagen (figure 37). The general morphology of the intermediate collagen appears to be arranged like screw threads or helical in shape (figures 37 and 38). The electron lucent space between the intermediate and the limiting membrane appears to be bridged by filaments and continuous, except at the region of direct exposure of the intermediate collagen and the predentin matrix (figure 37).

Extensive exposure of the intermediate with the predentin matrix is exhibited by the intermediate collagen in figure 39. From the exposed regions of the intermediate collagen, numerous banded collagen fibers appear to emanate directly and in a contiguous fashion from the intermediate collagen into the predentin matrix. In contrast to the extensive exposure of the intermediate collagen in figure 39, only a relatively small region of the intermediate collagen in figure 37 is directly exposed to the matrix. The remainder of the intermediate collagen in figure 37 appears within the limiting membrane and is similar in appearance to the extracellular intermediate collagen in figure 34.

b. Cytochemistry

Alkaline phosphatase activity was localized at the point of transition between the intermediate form of collagen and the banded collagen fiber in the predentin matrix (figures 37 and 39). Reaction product was evident at the periphery of the intermediate collagen as well as along the limiting membrane of the odontoblast. Enzyme activity is also present in

the predentin matrix and along the lateral membrane of the odontoblast.

A cloudy control specimen is shown in figure 38. A diffuse non-specific precipitate is evident both in the cell and in the extracellular space and this diffuse precipitate is in contrast to the localization of alkaline phosphatase reaction product in figure 37.

DISCUSSION

Predentin Matrix and Alkaline Phosphatase Activity

It has been well established that two developmentally distinct dentins, the initial mantle dentin and the subsequent circumpulpal dentin, are elaborated in the formation of tooth germ dentin. The former is highly limited in extent and develops only in the mesenchyme adjacent to the basal lamina of the enamel organ (78). The latter, circumpulpal dentin, continues from the mantle dentin and makes up the majority of the total dentin. Both types of dentin are preceded by the formation of an organic matrix which consists primarily of collagen fibers and secondarily of ground substance. In the circumpulpal predentin matrix, collagen has been shown to be the result of odontoblast synthetic activity (113). The origin of the collagen fibers of the mantle predentin matrix, in contrast, is unknown but is presumed to be derived from mesenchyme cells.

During dentinogenesis, mantle predentin matrix was shown in this study to differentiate from a relatively undifferentiated mesenchyme with sparse collagen fibers to a mature matrix with a dense collagen framework. The matrix collagen of mantle dentin was formed adjacent to the basal lamina of the enamel organ. Developmental changes in the differentiating predentin matrix were observable while concurrent changes in the distribution of alkaline phosphatase activity were also evident. Reaction product, associated with collagen fibers in the current study, was present only in the region of collagen deposition and the most recently formed fibers, but was not evident in the region of mature collagen fibers where calcification takes place.

Alkaline Phosphatase Electron Cytochemistry

Although the light microscopic location of alkaline phosphatase activity during dentinogenesis has been extensively described, few reported studies have dealt with the ultrastructural location of enzyme activity. Yoshiki and Kurahashi (120) described enzyme activity in the immature mantle predentin matrix and along the lateral membrane of the mature preodontoblasts. However, only a single developmental stage was represented in this study. Therefore, this relationship between matrix differentiation and alkaline phosphatase activity was difficult to establish. Leonard and Provenza (67) described alkaline phosphatase activity in the immature predentin matrix. Again, only a single developmental stage was represented in their study, and thus developmental changes in distribution were not described.

Larsson (61) described the distribution of alkaline phosphatase activity in mature mantle predentin matrix. He described intense activity in the dentin globules (matrix vesicles) as well as light deposition of reaction product dispersed throughout the predentin matrix. Reaction product was also demonstrated along the lateral membrane, but the developmental stage was not indicated. Larsson (61) used EDTA as an inhibitor of alkaline phosphatase in his control specimens in order to confirm the localization of enzyme activity.

The results of this study demonstrated that the distribution of alkaline phosphatase changes as a function of development during dentinogenesis. Reaction product in the mantle predentin matrix was shown to be faint at the initiation of development of predentin matrix. In later developmental stages, the intensity of the reaction product

increased. Reaction product was not a feature of the lateral membrane of the preodontoblast during mantle predentin matrix differentiation. However, it was found on the lateral membrane of the mature odontoblasts following the initiation of mantle dentin calcification and the formation of circumpulpal predentin matrix. The distribution of the reaction product was uniform in the early development of mantle predentin matrix but was strictly limited to the region adjacent to the mature odontoblasts and the most recently formed collagen fibers of the circumpulpal predentin matrix.

Collagen and Dentin Calcification

Following the initiation of mantle dentin calcification, the banded collagen fibers comprising the mantle predentin matrix become indistinct and the banding pattern is lost. The indistinct appearance of the mantle dentin matrix collagen is in sharp contrast to the distinct banded appearance of the collagen intimately associated with calcification of the circumpulpal matrix.

The banded appearance of collagen fibers is known to reflect the staggered arrangement of the polymerized tropocollagen molecules (106), and the bands are thought to represent periodic spaces between the linearly arranged tropocollagen molecules. Glimcher (40) recognized in bone that the periodic arrangement of the initial sites of calcification along the collagen fiber were similar to the banding pattern of the collagen. He hypothesized that calcification was dependent on the proper concentrations of the appropriate ions, including calcium and phosphate, within the periodic spaces or "hole zones" in the banded collagen fiber.

Evidence presented in the current study demonstrated a specific banding pattern of calcification along the von Korff fibers at the circumpulpal predentin matrix calcification front. The banding pattern associated with the von Korff fibers is similar in appearance to the "hole zone" calcification described by Glimcher (40). However, calcification of the remainder of the circumpulpal dentin does not follow this pattern. Generally, mantle dentin calcification, with matrix vesicles and indistinct collagen, may be distinguished from circumpulpal calcification with a lack of matrix vesicles and the presence of distinctly banded collagen fibers.

Matrix Vesicles

The appearance of matrix vesicles, apparently unique to the mantle predentin matrix, and their associated alkaline phosphatase activity was studied during dentinogenesis. Ultrastructural evidence demonstrated that matrix vesicles appeared during development, were restricted to the matrix adjacent to the basal lamina of the enamel organ, and disappeared during calcification of the mantle predentin matrix. The electron cytochemical evidence demonstrated that an increase in alkaline phosphatase reaction product was associated with the matrix vesicles during matrix differentiation.

It has been suggested that the vesicles may have a potential role in the inductive mechanisms between the enamel organ and the dental papilla. Croissant (24) described matrix vesicles in the early mantle predentin matrix and found RNA-protein complexes associated with the vesicles. Eisenmann and Glick (28) described a close spatial association between the vesicles and signs of initial calcification. They

postulated a role in calcification for the vesicles. The calcification associated with the vesicles could act as independent nucleation sites for the calcification of mantle dentin. Bernard (9) identified the "bud" or vesicles in the mantle dentin and noted that their disappearance correlated with the initiation of crystallization of the mantle dentin. The calcification was described as proceeding in a radial fashion outward from nucleation sites and the nucleation sites were proposed to originate from the matrix vesicles. Sisca and Provenza (94) stated that the vesicles in human deciduous teeth were located in the mantle predentin matrix. They suggested that the matrix vesicles originated from the differentiating odontoblasts and acted as initial sites of calcification.

Slavkin, H. C., P. Bringas, and R. Croissant (96) described the vesicles in the predentin matrix of the rabbit tooth germ in which RNA was shown to be associated with the matrix vesicles. More recently, it was suggested that the matrix vesicles were not homogeneous because their evidence indicated that only selected vesicles contain measurable amounts of RNA (97). They postulated that during tooth development a possible information transfer via the matrix vesicles occurs between the mesenchyme and the epithelium. This suggests a possible role for vesicles in the inductive mechanism known to occur between the developing tissues in the tooth germ, the dental papilla and the enamel organ (59). However, the precise nature of the influences of one tissue upon another tissue is not known.

Matrix vesicles were independently described by Anderson (5) and Bonucci (12, 13) in the calcification of cartilage. In the ultrastructural investigation of the initial intramembraneous bone formation,

Bernard and Pease (10) noted the presence of bone "nodules" from which calcification grew by coalesence. The nodules appeared to form crystal growth radially from the cellular extrusions of osteoblast origin.

Studying the calcifying long bone growth plate, Brighton and Hunt (19) demonstrated that matrix vesicles accumulated increasing quantities of calcium as the differentiating matrix approached actual calcification. The intravesicular calcium content appeared to parallel the stages of endochondral ossification and maximum content occured just prior to calcification. While the mitochondria contained maximum concentrations of calcium early in ossification, the matrix vesicles were devoid of measurable calcium. In contrast, the mitochondria appeared to lose calcium as the stages of endochondrol ossification proceeded. Brighton and Hunt (19) suggested that calcium may pass from the intracellular mitochondria to the extracellular matrix vesicles for subsequent nucleation of hydroxylapatite formation.

During the differentiation of mantle predentin matrix, the matrix vesicles exhibited increasing quantities of electron opaque material. For example, a matrix vesicle was noted in the early, immature mantle predentin matrix and it was devoid of electron opaque material. However, matrix vesicles in the mature mantle predentin matrix were inundated with electron opaque material, presumably consisting of enzyme reaction product and calcium phosphate crystals. Adjacent to the immature mantle predentin matrix, mitochondria were concentrated adjacent to the basal lamina in the undifferentiated inner enamel epithelium cells and within the mitochrondria numerous electron opaque particles were evident.

It was shown in the current study that as the inner enamel epithelium cells differentiated into the ameloblasts, the population of mitochondria decreased along the basal lamina cell surface of the ameloblasts and the electron opaque granules were not apparent. It is suggested that a similar phenomena occurs in dentinogenesis as that observed in endochondral bone ossification (19) in which the shifting of calcium from mitochondria to vesicles takes place prior to calcification. However, a distinct difference stands out concerning the tissue origin of the calcium. In bone, the mitochondria and the matrix vesicles are apparently of mesenchymal origin, while in dentinogenesis, the vesicles are of mesenchymal origin but the mitochondria are of an epithelial origin. It has not been proven that a shift of calcium from the epithelium mitochondria to the mesenchymal matrix vesicles actually occurs; however, based on the evidence from bone, it is possible that the initial calcification of the mantle dentin takes place through such a mechanism.

The biogenesis of the matrix vesicle has generally been regarded as a budding mechanism involving a portion of a cytoplasmic process which is pinched off from either an osteoblast or an odontoblast (83). However, this phenomenon has not been fully documented by electron microscopy. The tissue source of the vesicles has been postulated to be from the cells of the dental papilla and not the enamel organ (9, 96). It has been shown that during dentinogenesis the matrix vesicle membrane shows histocompatibility with the plasma membrane of the differentiating odontoblasts (97). This finding supports evidence that suggests a common origin of the vesicle membrane and the preodontoblast membrane.

In developing bone, alkaline phosphatase activity has been demonstrated in the matrix vesicles using electron cytochemical techniques (3, 7). In a single developmental stage of the predentin matrix, Larsson (61) demonstrated alkaline phosphatase activity in the matrix vesicles. Enzyme activity was localized on the vesicles in the mantle predentin matrix just prior to calcification. In addition, ATPase and Pyrophosphatase have been demonstrated cytochemically in the vesicles and may represent specific enzyme components of non-specific alkaline phosphatase (31). The role of ATPase activity in the matrix vesicle has not been determined (3). The matrix vesicle ATPase may act as a membrane pump mechanism which concentrates the calcium within the vesicle (2). It is suggested that the vesicles maintain an active transport of calcium. Vesiclar pyrophosphatase activity would presumably provide additional quantities of phosphate groups (30). Because elements necessary for calcification, i.e. calcium and phosphate and associated enzymes, appear closely related to the matrix vesicle, it has been suggested that the vesicles act as a nucleation site for hydroxylapatite formation (9, 20).

Enzyme Components of Alkaline Phosphatase

Alkaline phosphatase may function as a nonspecific enzyme because a variety of phosphate substrates are susceptable to the catalytic activity of the enzyme. Pyrophosphatase and ATPase have been identified as specific enzyme components of purified nonspecific alkaline phosphatase that was extracted from bone and isolated by starch gel electrophoresis (24 & 25). Morphologically, distinct localization of alkaline phosphatase activity may reflect the functional location of the specific components of the nonspecific enzyme. The specific and distinct localiza-

tions of alkaline phosphatase activity shown in the current study, i.e., within the matrix vesicles, on the lateral membranes of the odontoblasts, associated with collagen fibers, at the periphery of the intermediate collagen, and dispersed in the mantle predentin matrix, may represent different, specific enzyme components. While each component appears to have a pH optimum for the respective substrate, the interrelationship between nonspecific alkaline phosphatase and ATPase and pyrophosphatase has not been determined.

a. Pyrophosphatase Component

Woltgens, J.H.M., S.L. Bonting, & O.L.M Bijvoet (119) assayed alkaline phosphatase enzyme activity from mature odontoblasts which were dissected from the adjacent circumpulpal predentin matrix. Alkaline phosphatase activity from mantle dentin was not included in the assay. The biochemical results indicated that pyrophosphatase activity was part of the nonspecific alkaline phosphatase activity. Using electron cytochemical techniques, Larsson and Helander (63) identified pyrophosphatase activity in the circumpulpal predentin matrix. Thus, pyrophosphatase activity is present in both mantle (3, 7), described previously, and circumpulpal predentin matrices prior to calcification, i.e., associated with the vesicles in the former and dispersed in the matrix in the latter. In the current study, alkaline phosphatase activity was evident in the matrix vesicles and was dispersed in the circumpulpal predentin matrix. This distribution of nonspecific alkaline phosphatase activity may represent the specific pyrophosophatase component. The difference in the distribution of the enzyme may represent a difference in the mechanisms of calcification between the two dentin matrices.

b. ATPase Component

In addition to the pyrophosphatase activity, it has been shown that nonspecific alkaline phosphatase in developing bone includes an ATPase component (30). This ATPase was not a true calcium-magnesium activated ATPase as compared to the ATPase found in the intestine, but was, nevertheless, responsive to calcium (89). Heyden and Fromme (49, 50) demonstrated ATPase activity histochemically in the odontoblast cell layer in the developing tooth, although the precise localization of this enzyme was not determined. They noted that the onset of ATPase activity, associated with the odontoblasts, corresponded with the onset of calcification of the mantle dentin. It was noted in the present study that reaction product was localized on the lateral membrane of the odontoblast after evidence of calcification of the mantle dentin was apparent. The acquisition of phosphatase activity on the lateral membrane may be related to the onset of calcification.

Membrane associated ATPase activity generally functions in active transport and the utilization of energy (71). It has been shown that bone cells adjacent to regions of calcification may accumulate calcium ions (74). Fromme (37) presented autoradiographic evidence in support of the hypothesis that calcium passes through the odontoblast en route to the predentin matrix and subsequent calcification. This evidence suggests that the reaction product along the lateral membrane of the odontoblast may in fact represent the ATPase component of tooth germ alkaline phosphatase. The calcium would course from the subodontoblast capillary plexus into the odontoblast via the lateral membrane. The calcium would then pass into the predentin matrix via the apical membrane

for subsequent calcification. Based on the possible relationship between the lateral membrane reaction product and the onset of mantle dentin calcification presented in the current study, it is suggested that the reaction product on the membrane represents a membrane associated pumping mechanism for the accumulation of calcium in the odontoblast. Hence, reaction product of alkaline phosphatase activity localized in different regions of the odontoblast may be correlated with different functions.

Collagen Elaboration and Alkaline Phosphatase Activity

The mature odontoblast is responsible for the elaboration of the circumpulpal matrix. Enzyme reaction product was associated with the most recently formed collagen fibers during this elaboration. The evidence in the present study indicates a possible relation between enzyme activity and the extrusion of collagen from the odontoblast.

The collagen fibers intimately associated with immature mesenchymal cells had little demonstrable alkaline phosphatase activity and this is in marked contrast with the notable reaction product associated with the fibers being extruded from maturing odontoblasts. This difference may be the result of matrix and odontoblast differentiation. Linsenmayer, et al., (70) demonstrated that in chick limb bud development the molecular structure of collagen changed as the tissues differentiated. Thus, collagen composition may be altered as a function of development. The appearance of alkaline phosphatase activity associated with collagen formation in the mature odontoblast may reflect a developmental change in collagen synthesis during the differentiation of the odontoblast from the mesenchyme cell.

The observed difference in alkaline phosphatase activity between collagen extruded by the mature odontoblast and extruded by the mesenchyme cell progenitor may reflect a difference in the phosphate content of the two developmentally distinct collagens. Schleuter and Veis (92, 110) demonstrated a quantitative difference in phosphate content of dentin collagen compared to skin collagen. Phosphate groups were found to be bound to the dentin collagen but were absent in skin collagen produced by fibroblasts. Although the significance of the phosphate groups in dentin collagen is not known, it has been postulated that the phosphates may be involved in the extracellular assembly of collagen fibers (111).

The majority of the phosphate groups in dentin were shown by electrophoresis to be bound to a noncollagen phosphoprotein (109). A fast migrating component, isolated from dentin collagen, was composed of a noncollagen phosphoprotein covalently bound to a collagen polypeptide backbone (20). Veis and Schleuter (110) hypothesized that dentin collagen contains additional inter- and intramolecular cross links in comparison with skin collagen. The extra cross links were believed to contribute strength and stability to the dentin matrix collagen. Rubin (88) also suggested noncollagen groups may participate in the collagen cross linkage. Involvement of the phosphate groups in collagen fibrillogenesis was hypothesized to occur very early in the extracellular polymerization of collagen (111).

If phosphate groups are involved in the crosslinking of extracellular collagen fibers in the predentin matrix, then the question arises as to where, in the biosynthetic pathway of dentin collagen, are phosphate groups incorporated into the crosslinks. It is not known if phosphate groups are present in the intracellular procollagen. However, it is

known that the phosphate groups are present in extracellular polymerized tropocollagen (92). In the present study, alkaline phosphatase activity in the circumpulpal predentin matrix was demonstrated to be associated with both the most recently formed fibers and the banded collagen fibers just as they emerged from the collagen intermediate. This evidence suggests the availability of free phosphate ions in the region of initial banded collagen fiber formation. Thus, an association between alkaline phosphatase activity and extracellular collagen fiber formation during dentinogenesis was hypothesized. It is suggested that alkaline phosphatase activity represents a potential source of phosphate groups in the extracellular matrix. However, the precise role of alkaline phosphatase in collagen biosynthesis remains speculative.

Collagen Biosynthesis

The elaboration of predentin collagen by the odontoblast has been verified morphologically (38, 75, 94, 101, 102, 114, 117) as well as autoradiographically (4, 22, 54, 84, 116) and with histochemistry (117). The polarization of the odontoblast provides an excellent model of collagen biosynthesis because of its highly predictable point of deposition near the apical end of the cell, and the sequential route of collagen metabolites passing from the cell into the predentin matrix.

The biochemical features of collagen biosynthesis have been reviewed recently and are briefly summarized (47, 48, 72, 93, 106). The nascent, procollagen alpha chains are synthesized on the ribosomes and a noncollagen molecular extension is attached to the amino terminal end. These extensions have been shown to contain disulfide bonding which is believed to orient the individual alpha chains into the triple helix

characteristic of procollagen molecules. The triple helix plus the molecular extension are estimated to be about 320 nm in length.

Glycosylation of procollagen helices is believed to occur in the Golgi, and the number and kinds of sugars attached to the residues of the triple helix contribute to the characterization of the various types of collagen. In addition to glycosylation, the condensing and packaging of the triple helix is hypothesized to occur in the Golgi. The intracellular transport form of collagen, procollagen, is immunoactive, while the extracellular, polymerized collagen is not. Immunotechniques adapted for electron microscopy (81) have shown procollagen localized in the Golgi saccules of fibroblasts (79). The packaged helix and extension are then transported to the site of extrusion, which, in the case of the odontoblast, is near the apical end of the cell next to the predentin matrix (113).

In the odontoblast, the Golgi region has been shown to play a role in the biosynthesis of collagen. Autoradiographic investigations using labeled collagen precursors such as ^{3}H-proline and histochemical investigations using silver methenamine as a specific glycoprotein stain for collagen or low pH phosphotungstic acid (117) have established the presence of the collagen precursors in the Golgi. Early procollagen synthesis within the Golgi of the odontoblast using a high pH lead stain was established by Weinstock (115).

Procollagen is transformed to tropocollagen when it leaves the cell but it has not been determined exactly where this process occurs. The noncollagen molecular extension is cleaved from the three chain helix by a neutral protease, procollagen peptidase (15, 42, 43, 58).

Since small amounts of procollagen (42) and procollagen peptidase (14, 58) have been found extracellularly, it is hypothesized that the enzymatic activity occurs extracellularly. The tropocollagen helices, about 280 to 300 nm long, then polymerize extracellularly to form typical banded collagen fibers.

The Odontoblast and Collagen Biosynthesis

During the extrusion of collagen from the cell, the transition from intra- to extracellular location is a poorly defined phenomenon (14). The cellular mechanism by which the procollagen moves through the odontoblast from the Golgi complex and is deposited in the matrix as tropocollagen also is a subject of controversy.

The evidence of Frank (34), Weinstock (114, 115), and Weinstock and Leblond (117) demonstrated that the procollagen contained within secretory "granules" originates from the Golgi and apparently migrated to the apical cytoplasm of the odontoblast. The secretory "granule" was described as oblong or cigar-shaped and contained electron opaque particles. Neither the orientation of the procollagen within the secretory "granules" nor the relation of the electron dense particles to the procollagen were established. These authors promulgated a process of exocytosis in which the individual granules fuse with the plasma membrane of the odontoblast. Subsequently, the procollagen was extruded into the predentin matrix in the form of tropocollagen molecules. The general implication of this process involved numerous secretory granules releasing their molecules, which would then individually polymerize into unique and continuous banded collagen fibers.

In contrast, Reith (54, 84) and Kajikawa and Kajihara (54), also using

^3H-proline isotope for autoradiography, found little or no evidence of the procollagen isotope associated with the secretory granules. Reith (84) suggested that the collagen molecule exits from the cell through the plasma membrane into the predentin matrix. Their hypothesis included membrane alterations as the collagen molecule slipped through. The ultra-structural evidence indicated a close relationship between the extra-cellular collagen and the membrane of the odontoblast, but an intracellular relationship was not well defined.

In either of the above cases, the evidence for the extrusion of the collagen molecule from the odontoblast is not complete. Numerous investigators have described the membrane bound secretory "granules" in the apical cytoplasm of the odontoblast (38, 62, 75, 84, 84, 101, 102) but membrane fusions and figures resembling exocytosis were rarely if ever demonstrated and, therefore, the "granules" were discounted as a possible source of the dentin collagen (75, 84 & 94). Thus, a paradox exists between the strong evidence for the collagen molecule within the secretory "granules" and the essential lack of evidence for the participation of the secretory "granules" in exocytosis which would account for the extrusion of collagen. Weinstock and Leblond (119) believe the process is rapid enought to avoid catching many at one time. Whether this can explain this essential lack of evidence for exocytosis is debatable.

The transition from the intracellular procollagen secretory package to the intermediate form of collagen appeared to involve an interaction between two distinct morphological entities, the secretory package and the membrane bound vesicle. Specific morphological changes noted in the current study, i.e., the development of an electron lucent space

and the apparent loss of the electron opaque particles, apparently result from this interaction. It may be that the enzyme, procollagen peptidase, possibly originating in the vesicles, passes through the electron lucent space into the predentin matrix. This would account for the biochemical evidence which indicated that procollagen peptidase was extracellular (15 & 58).

The electron opaque particles may represent lead positive staining by the lead in the reaction mixture of the condensed disulfide groups known to be in the noncollagen molecular extension. If the cleavage of the molecular extension from the tropocollagen helix by procollagen peptidase results in the disperson of the condensed noncollagen molecular extension, then this enzymatic activity may account for the disappearance of the electron opaque particles.

Intermediate Collagen

The absence of electron opaque particles and the appearance of a continuous electron lucent space between the secretory package limiting membrane and the collagen contents are characteristic features of the intermediate form of collagen.

An intermediate form of collagen was identified ultrastructurally in the current study and was observed consistently at the apical end of the odontoblast cell layer, usually between adjacent odontoblasts. Continuity between the intracellular procollagen and the extracellular banded collagen fiber was accomplished by the intermediate collagen. Recent biochemical studies have established the existence of a possible intermediate form of collagen which may be involved in the initial polymerization of the banded collagen fibers (23, 42, 43, 107 & 108). How-

ever, the biochemical nature of the intermediate form of collagen was not determined.

Collagen Extrusion

If procollagen is the major constituent of the secretory "granules" or "packages," a number of explanations of the mode of the extrusion of the collagen molecules are possible. In order to identify and describe the process of collagen extrusion, a description of the intracellular collagen is necessary in order to establish the continuity between the intracellular and extracellular collagen. The intracellular secretory package appeared in a variety of configurations. Elongated and tubular intracellular collagen packages (1000 nm and longer) were noted in the current study. Short, oblong secretory packages (300-400 nm long) and apparent cross sectional diameters (less than 100 nm) were also shown. Although the length of the secretory package may be a determining factor in the extrusion process, the longitudinal morphology of the package has not been fully described.

First, the intracellular secretory "granule" or "package" can appear to exist as a single, oblong package. Second, the secretory package can appear elongated or tubular as if a number of the individual "granules" had fused end to end in a longitudinal fashion. Third, any or all of the secretory packages may be connected in a single or a number of long tubes, with the packages representing only that portion within the particular plane of section.

The extrusion of collagen from the odontoblast (117) and other collagen producing cells (106) has been hypothesized to occur via exocytosis. This mechanism was modeled after discharge of zymogen from

intracellular granules into the lumen in the pancreatic acinar cells (71). However, zymogen consists of globular soluble proteins and the comparison of the extrusion of globular proteins with the extrusion of fibrous proteins may not be justified. It is difficult to verify the extrusion of collagen from the odontoblast via exocytosis without ultrastructural evidence of such a mechanism.

The mechanism of exocytosis implies the fusion of the package membrane with the apical membrane and the subsequent release of the individual procollagen molecules into the matrix. The question of where procollagen is converted to tropocollagen in the mechanism of extrusion by exocytosis has not been determined. Also, the cellular site of procollagen peptidase activity is not known.

The extracellular assembly of tropocollagen units into banded collagen fibers results in a unique fiber, or group of fibers, characteristic of the elaborating tissue. If the individual tropocollagen molecules are liberated into the extracellular predentin matrix via exocytosis of the individual "granules," then this extracellular polymerization would form the characteristic fibers randomly and the polymerization would conform to the size and number of fibers found in the predentin matrix. However, the evidence in this study suggests that the intermediate form of collagen appears to be the result of conversion from the intracellular procollagen. Then, the characteristic size and number of banded collagen fibers are contiguously elaborated from the intermediate collagen into the predentin matrix. In this way the intermediate form of collagen would act as an organizing center to provide the unique size and number of banded fibers for these collagen producing cells.

SUMMARY AND CONCLUSIONS

Dentinogenesis is a process in the developing tooth germ which involves the sequential elaboration and maturation of dentin. This study considered two aspects of dentinogenesis. First, the development of both mantle and circumpulpal predentin matrices was examined, and second, the extrusion of collagen from the mature, polarized odontoblasts was studied.

The cellular and extracellular events which occur during the sequential development of the predentin matrices were established ultrastructurally. The relationship between a specific stage of predentin matrix formation and the presence of alkaline phosphatase activity was determined. During mantle dentin formation, reaction product was associated with the matrix vesicles and dispersed evenly in the matrix. During circumpulpal dentin formation, however, reaction product was associated with the lateral membrane of the mature, secretory odontoblast and the newly synthesized collagen in the predentin matrix adjacent to the odontoblast.

An intermediate form of collagen was defined, and a novel cellular mechanism for the extrusion of collagen from the odontoblast was hypothesized. It is suggested that intracellular collagen is organized in membrane bound "packages" in the Golgi and is transported to the junctional region of the lateral membrane. Following the intracellular interaction of the "package" with the electron lucent vesicle in the formation of the intermediate collagen, the membrane of the collagen fuses with the lateral membrane. Alkaline phosphatase activity is first associated with the extrusion process when the intermediate collagen reaches the lateral membrane. Banded collagen fibers emanate directly from the intermediate collagen into the predentin matrix.

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ILLUSTRATIONS

All preparations for electron microscopy were incubated for alkaline phosphatase activity except the noted substrate free and cloudy controls. The specimens were stained with both uranyl acetate and lead citrate. The following is a list of abbreviations.

Am	Ameloblasts
AC	Apical Cytoplasm
BL	Basal Lamina
Co	Banded collagen fibers
CP	Cell Process
Den	Dentin
DL	Dental lamina
DP	Dental Lamina
ELS	Electron Lucent Space
En	Enamel
EOP	Electron Opaque Particles
G	Golgi
J	Junction between odontoblasts
ICo	Intermediate Collagen
IEE	Inner Enamel Epithelium
LE	Leading Edge of the enamel organ
LM	Lateral Membrane of the odontoblast
Ma	Matrix
MC	Mesenchymal Cell
Mi	Mitochondrion
MV	Matrix Vesicle
N	Nucleus
nm	nanometer
Od	Odontoblast
OE	Oral Epithelium
OEE	Outer Enamel Epithelium
OP	Odontoblast Process
PAm	Pre-ameloblasts
POd	Pre-odontoblast

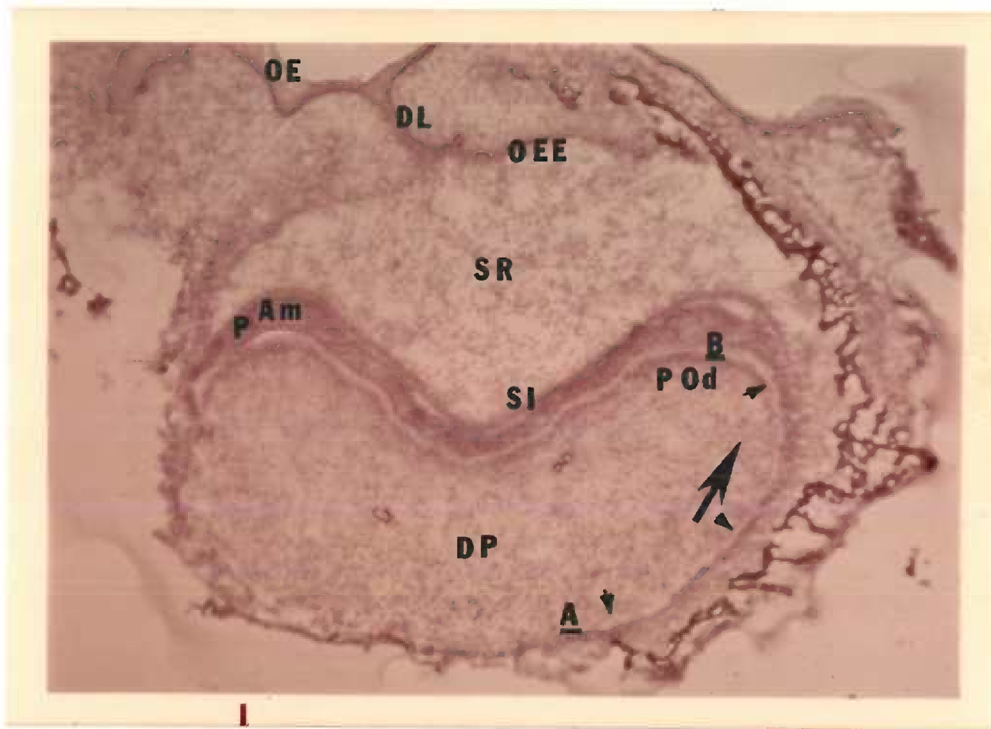
RER	Rough Endoplasmic Reticulum
RP	Reaction Product of alkaline phosphatase activity
Sac	Golgi Saccule
SI	Stratum Intermedium
SubOd	Subodontoblasts
SP	Secretory Package
SR	Stellate Reticulum
V	Vesicle
vK	von Korffs fibers

Figure 1. Neonate rat first molar tooth germ in cross section.

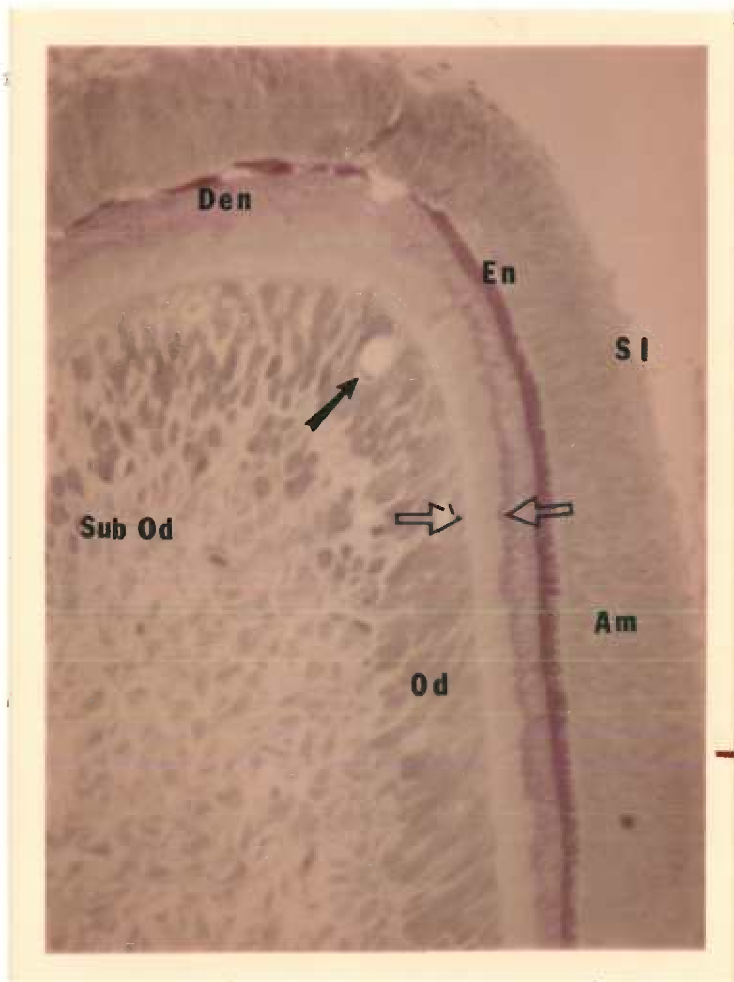
Cusp development is evident in the specimen and the elements of the enamel organ; the inner enamel epithelium (IEE) which gives rise to preameloblasts (PAm), the stratum intermedium (SI), the stellate reticulum (SR), and the outer enamel epithelim (OEE), are attached to the oral epithelium (OE) via the dental lamina (DL). The dental papilla (DP) includes the mesenchyme within the confines of the enamel organ and neither calcified dentin nor enamel are apparent. The mantle predentin matrix (arrow heads) develops in the mesenchyme adjacent to the IEE, and forms a developmental continuum from point A to point B (arrow). The former represents the least differentiated matrix at the area of initial interaction between the leading edge (LE) of the enamel organ and the undifferentiated mesenchyme. The latter, point B, represents the area of greatest differentiation in the region of the cusp. Alkaline phosphatase substrate free control specimen, modified Gomori, Mayers Hematoxylin counter stain. (64X)

Figure 2. Two day old rat first molar tooth germ: cross section of the cusp region.

The cusp region of the 2 day old rat first molar exhibits both enamel (En) and dentin (Den). The circumpulpal predentin matrix (open arrows) is sandwiched between the mantle dentin (DEN) and the polarized odontoblasts (Od). A capillary (arrow) extends into the odontoblast layer. The majority of the papilla including the subodontoblasts appear similar to mesenchyme. Histological preparation. (96X)



OE
DL
OEE
SR
P
Am
B
POd
SI
DP
A



Den
En
SI
Sub Od
Am
Od

Figure 3. Histochemistry of the leading edge of the enamel organ and adjacent mesenchyme.

This alkaline phosphatase histochemical preparation represents an area similar to the one marked A in figure 1. It includes a portion of the mesenchyme of the dental papilla (DP) adjacent to the leading edge (LE) of the enamel organ. The edge represents the point of differentiation between the IEE and OEE. The adjacent mesenchyme (open arrow) represents the point of initiation of the mantle predentin matrix. Alkaline phosphatase activity, a red precipitate, is most noticeable in the developing enamel organ, and it is absent or faint in the DP. Alkaline phosphatase; substrate AS BI-phosphate, azo dye Fast Red TR, Methyl green counter stain. (96X)

IEE
OEE
LE
DP

Figure 4. The tip of the leading edge and the initial mantle predentin matrix.

An ultrastructural overview of the initial mantle predentin matrix is shown adjacent to the leading edge of the enamel organ. The space between the basal lamina (BL) of the enamel organ and the mesenchyme cell (MC) forms the presumptive mantle predentin matrix. Collagen fibers appear dispersed but are organized into groups in the extracellular space. Numerous cell processes are evident in the mesenchyme. The mesenchyme cell appears to be extruding collagen fibers (arrow in rectangle). Alkaline phosphatase activity, represented by the electron opaque particles, appears faint or absent in the mesenchyme. (14,250X)

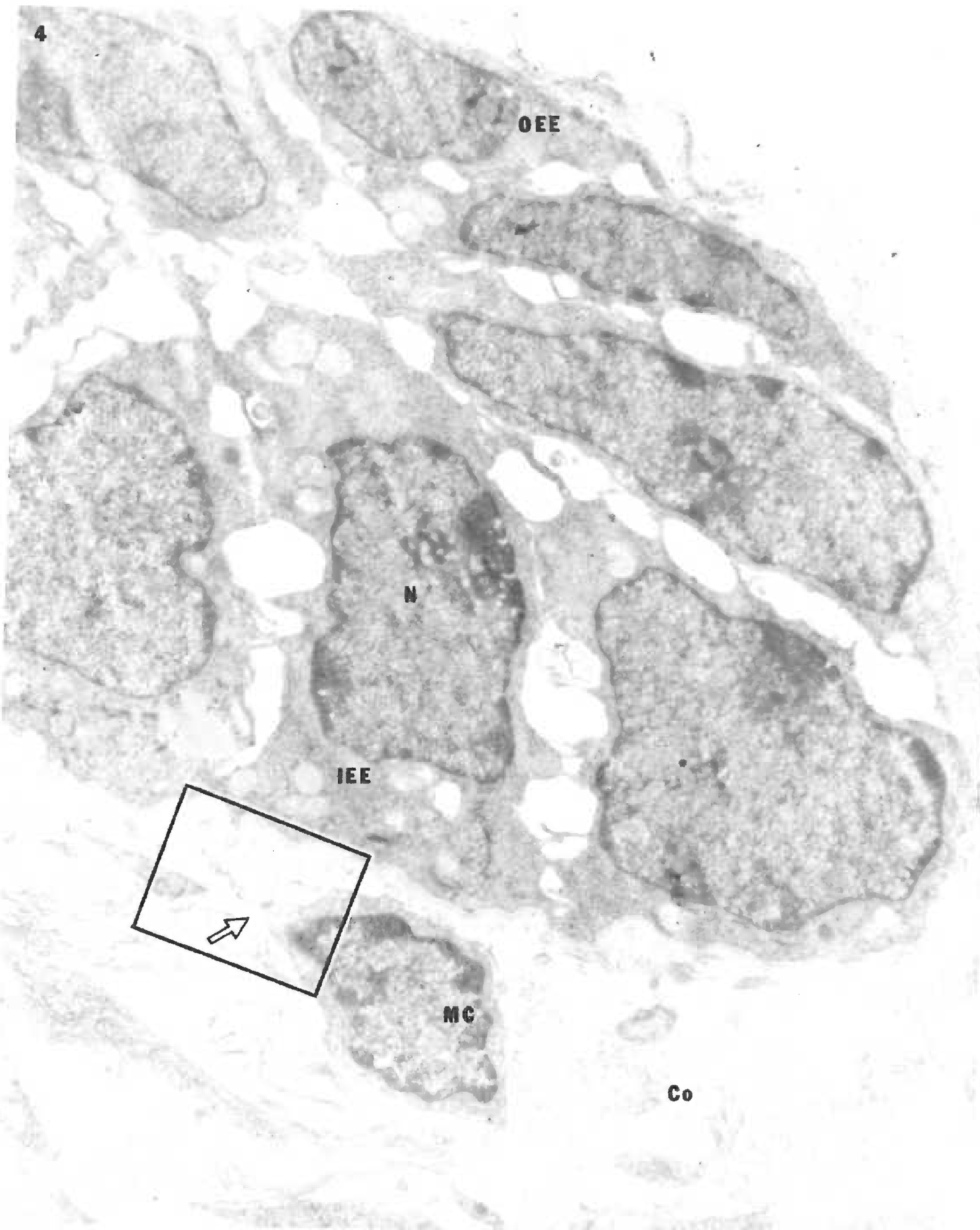

4
OEE
N
IEE
MC
Co

Figure 5. The initial stage of the mantle predentin matrix development.

The rectangle in figure 4 is shown at higher magnification and represents the fine structure of the initial mantle predentin matrix. The matrix is limited by the basal lamina of the IEE. From the basal lamina, nonbanded collagen (Co) fibers penetrate the matrix space. The mesenchyme cell appears to be extruding collagen fibers which appear as banded fibers in the matrix space. Alkaline phosphatase reaction product appears absent or lightly dispersed in the mesenchyme extracellular space. (85,000X)

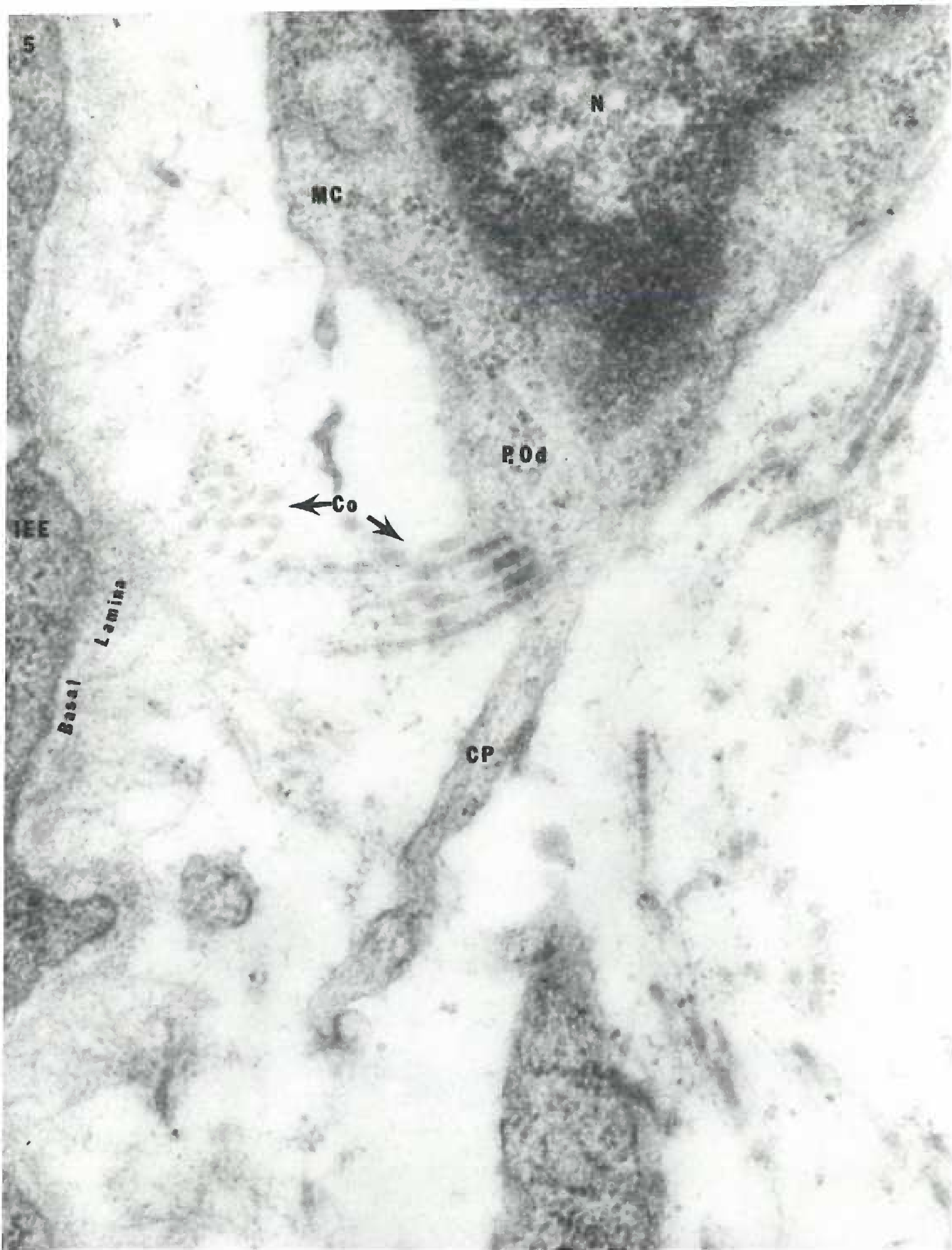

5
N
MC
P.Od
Co
IEE
Basal Lamina
CP

Figure 6. The enamel organ and adjacent mesenchyme with a distinct mantle predentin matrix space.

In this histochemical preparation, a region of the immature predentin matrix (about midway between A and B in figure 1) is shown. The preameloblasts (PAm) of the enamel organ appear continuous along the matrix space and there is an intense deposit of red reaction product in the developing stratum intermedium (SI). Light, dispersed reaction product is evident in the matrix space as well as in the mesenchyme of the dental papilla (DP). Alkaline phosphatase; substrate AS BI-phosphate, azo dye fast Red TR, counter stain Mayers Hematoxylin. (190X)

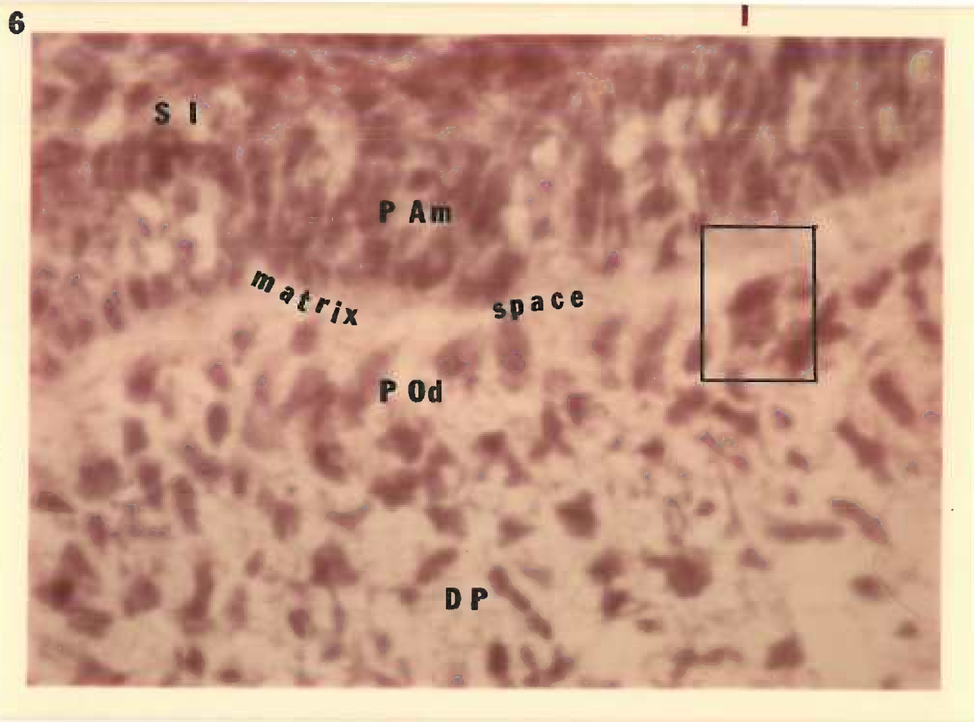
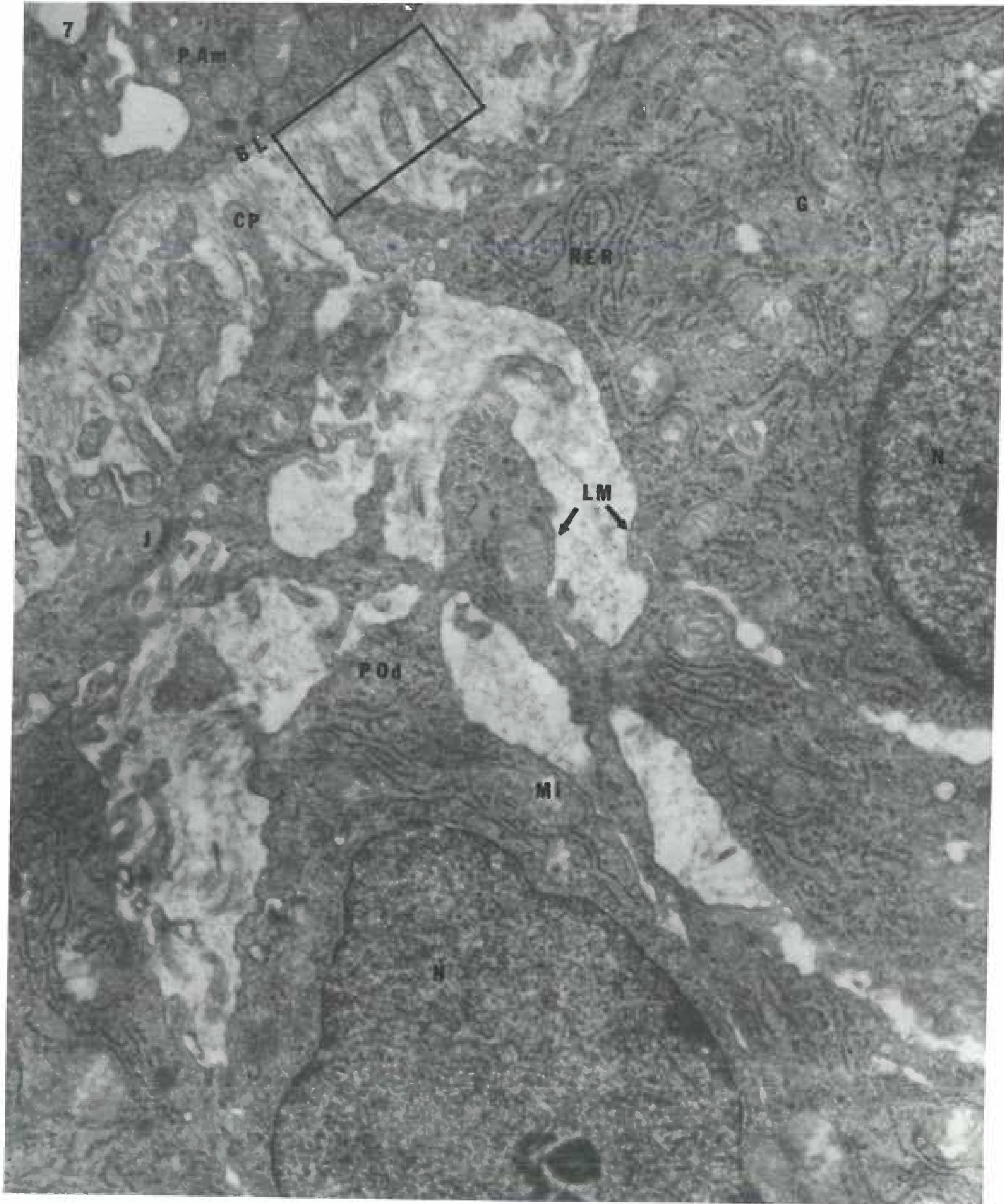

6
S I
P Am
matrix
space
P Od
DP

Figure 7. The immature, mantle predentin matrix and adjacent preodontoblasts.

This overview electron micrograph shows the relationship between the preameloblasts (PAm) of the enamel organ and the basal lamina (BL), and immature mantle predentin matrix and the preodontoblasts (POd). The figure represents a stage of development comparable to that found about midway between A and B in figure 1. The basal lamina forms a continuous boundary along the matrix space and sends fibers into the space. Attenuated cell processes (CP) from the apical end of the preodontoblasts (POd) also penetrate the matrix space perpendicular to the basal lamina. Mitochondria (Mi), rough endoplasmic reticulum (RER), and Golgi elements (G) are all evident in the preodontoblasts (POd). Junctions (J) are evident as are the lateral membranes (LM) of the preodontoblasts. (17,750X)



7
PAm
BL
CP
RER
G
N
LM
J
POd
Mi
N

Figure 8. The immature mantle predentin matrix.

The fine, collagen fibers (perpendicular to the thick arrows) emanating from the basal lamina are shown interspersed with banded collagen fibers (Co), of mesenchyme origin, in the immature mantle predentin matrix space. This region is comparable to the outlined region in figure 7. Membrane limited matrix vesicles (MV) are evident in the space among the collagen fibers as are cell processes (CP) which originate from the apical end of the preodontoblast (POd). The matrix vesicles (MV), not apparent at the initiation of the predentin matrix, appear to contain varying amounts of electron opaque material. Alkaline phosphatase activity is represented by the dispersed electron opaque particles, associated with collagen fibers, both banded and nonbanded, with the cell processes, and with the electron lucent ground substance. (61,500X)

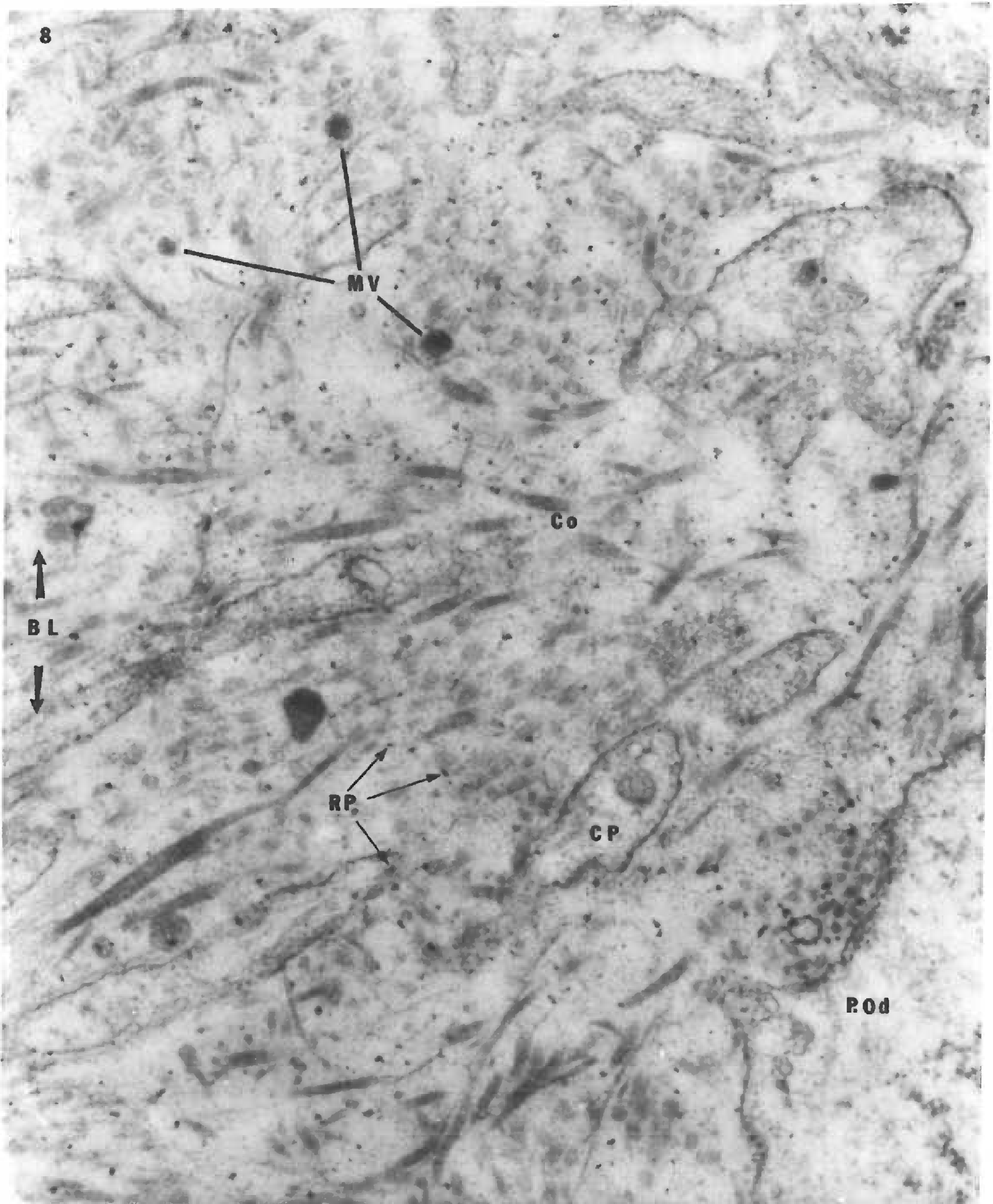

8
MV
Co
BL
RP
CP
P.Od

Figure 9. Matrix vesicles in the mantle predentin matrix.

Two matrix vesicles (arrow heads) in the immature mantle predentin matrix are shown at high magnification. The tri-laminar membrane is evident as is electron opaque material within the vesicles. The electron opaque material presents two characteristic images; either thin and elongated, or rounded and globular. Alkaline phosphatase activity is represented by the electron opaque, globular images (arrows) in the matrix. (375,000X)

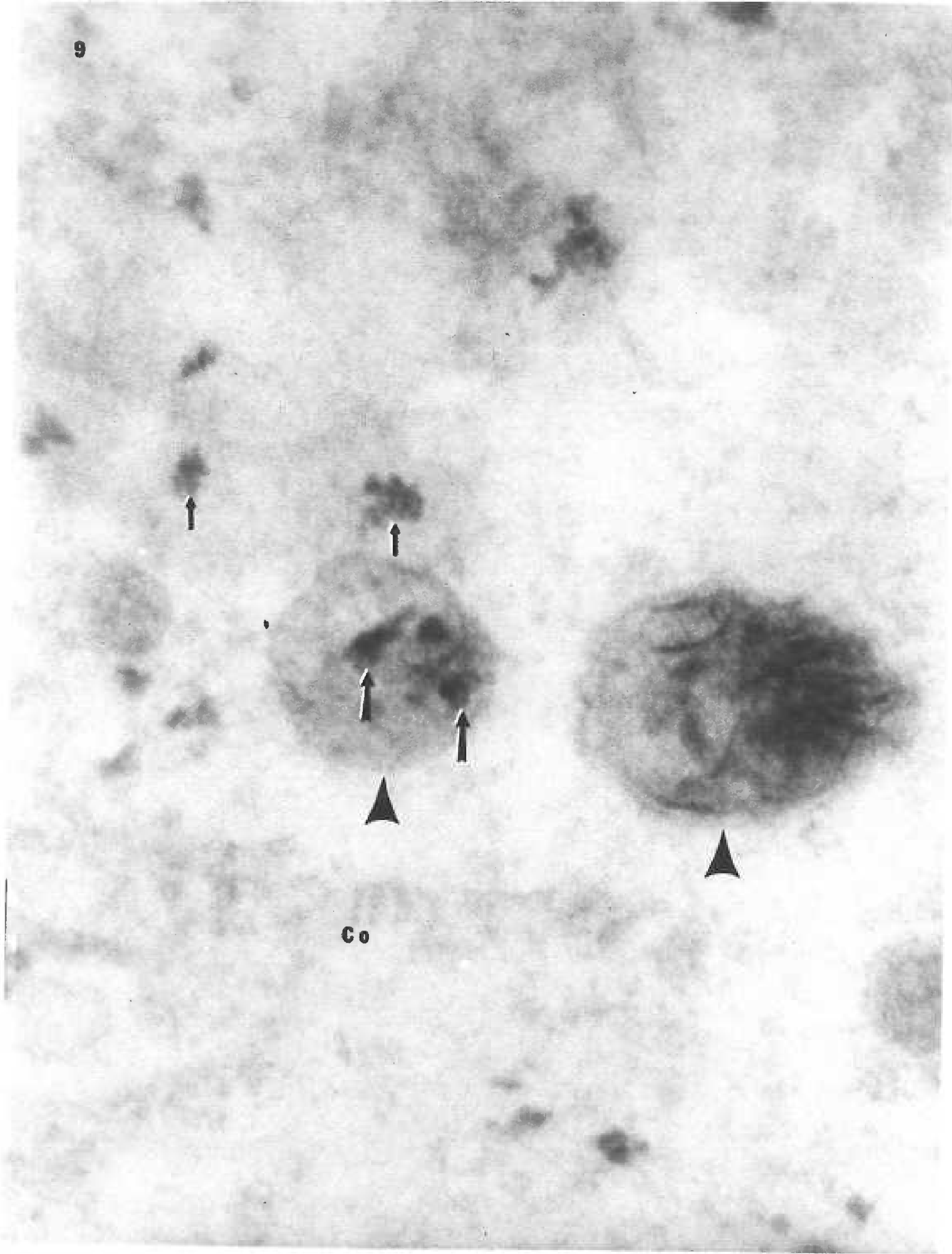

9
Co

Figure 10. A developmentally early, immature mantle predentin matrix.

A very early, immature predentin matrix is represented in figure 10. The width of the mantle predentin matrix is illustrated from the surface of the preodontoblast (POd) to the basal lamina (BL) of the inner enamel epithelial cell (IEE). The collagen fibers appear faintly banded and they frequently appear in groups. The inner enamel epithelial cell (IEE) is shown with numerous mitochondria (Mi) between the nucleus (N) and the basal lamina surface of the plasma membrane adjacent to the matrix. Electron opaque particles are numerous within the mitochondria. Alkaline phosphatase activity appears primarily associated with fibrillar elements in the mantle predentin matrix and the basal lamina of the enamel organ. (61,500X)

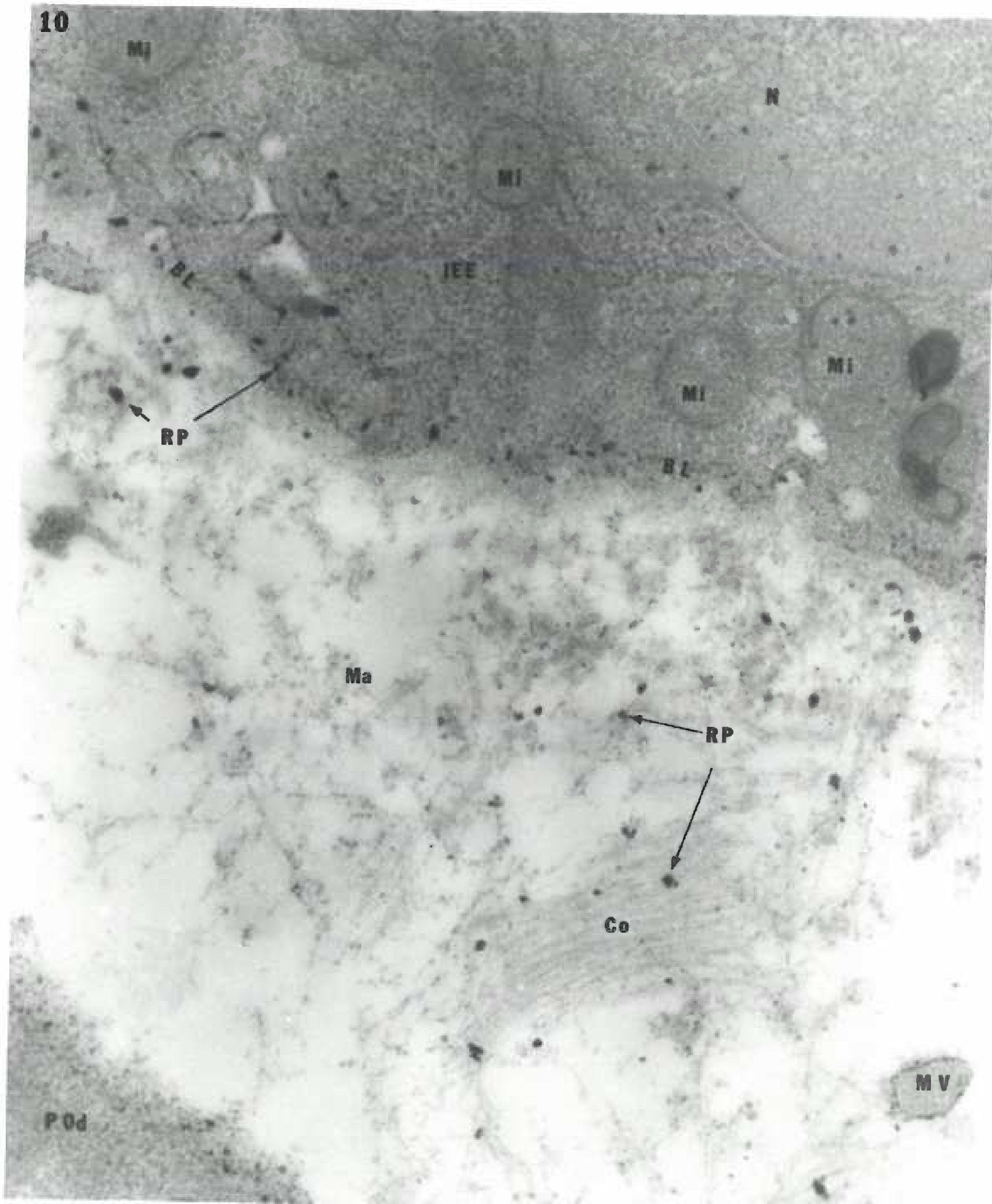

10
Mi
N
Mi
BL
IEE
Mi
Mi
RP
BL
Ma
RP
Co
MV
POd

Figure 11. The mature mantle predentin matrix and preodontoblasts.

The mature mantle predentin matrix (Ma) is clearly demarcated by the basal lamina (BL) of the enamel organ and the polarized preodontoblasts. Numerous and branching cell processes (CP) are shown extending into the mature matrix. Electron opaque particles are identifiable in the matrix, while alkaline phosphatase activity is faint at best in this low magnification electron micrograph. The nucleus (N) occupies the basal region of the cell while the rough endoplasmic reticulum (RER), Golgi (G) and mitochondria (Mi) saturate the cytoplasm (supranuclear cytoplasm) between the nucleus (N) and the apical cytoplasm of the cell. (6,500X)

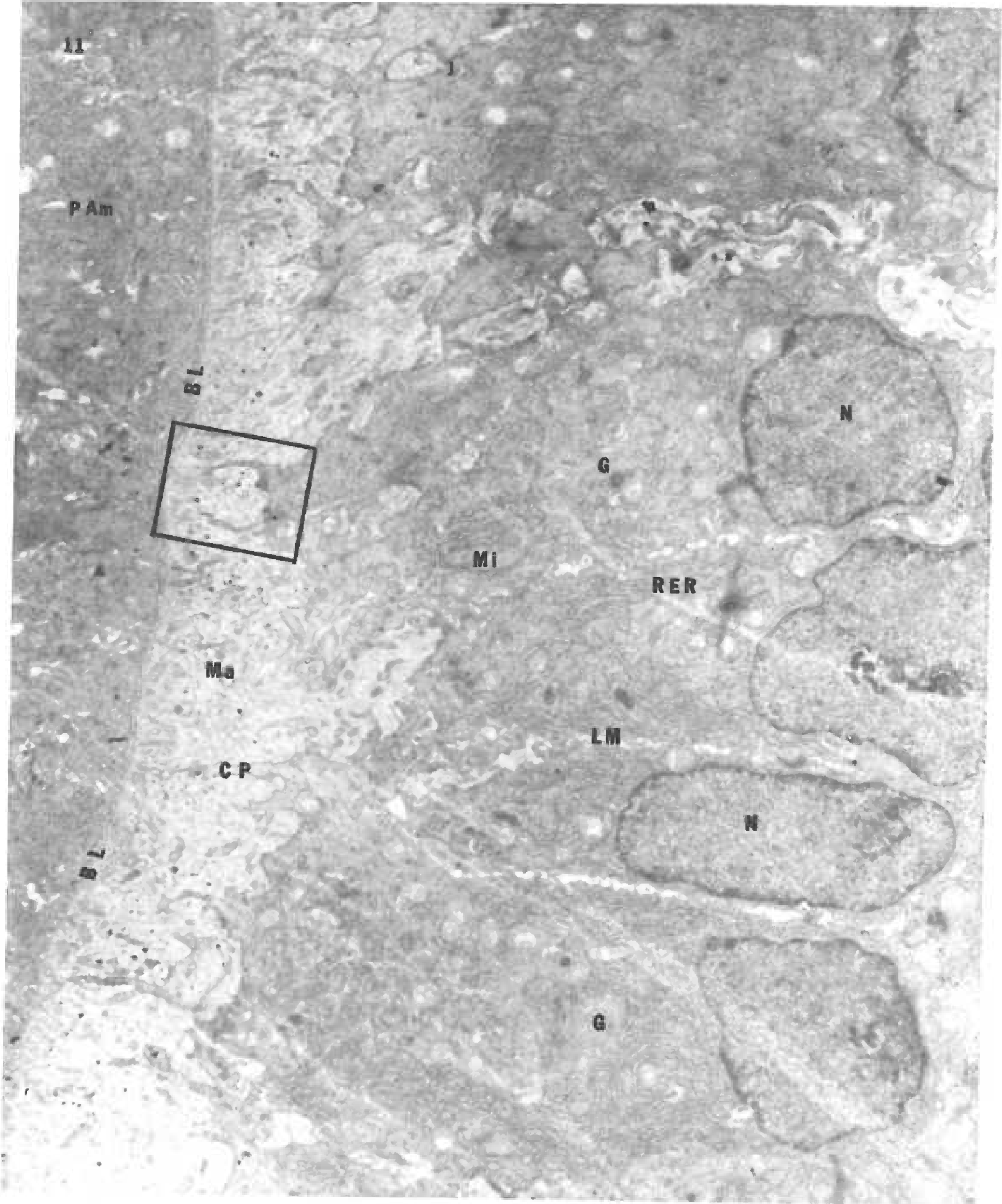

11
PAm
BL
N
G
Mi
RER
Ma
LM
CP
N
BL
G

Figure 12. The mature, mantle predentin matrix.

The region shown in this figure is comparable to that outlined in the rectangle in figure 11. This represents a tangential section through the matrix and demonstrates irregularities in the basal lamina (BL). The apical cytoplasm (AC) of several preodontoblasts (POd) extend into the matrix space. Collagen fibers (CO) and cell processes (CP) are evident throughout the extent of the mature matrix, while the matrix vesicles (arrowhead) are stationed near the basal lamina (BL). Alkaline phosphatase activity is represented as a fine granular precipitate throughout the matrix (arrows) but not within the odontoblast proper nor associated with the plasma membrane. Within the preodontoblast supranuclear cytoplasm, rough endoplasmic reticulum (RER) and mitochondria (Mi) are evident as are secretory packages (SP). However, the apical cytoplasm (AC) remain devoid of the major organelles. Junctions (J) are a prominent feature of the plasma membranes between adjacent preodontoblasts. (14,250X)

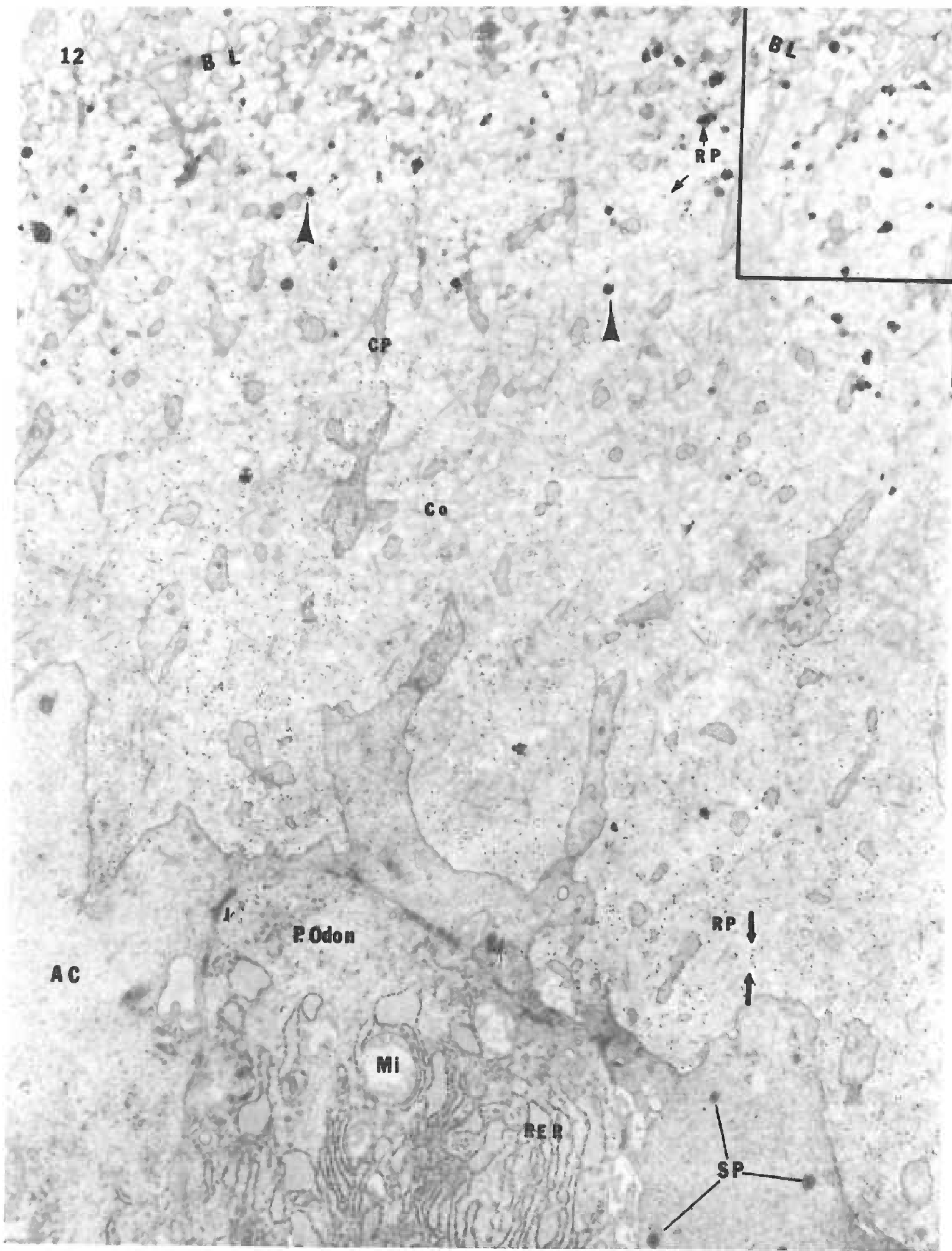

12
BL
BL
RP
CP
Co
P. Odon
AC
RP
Mi
RER
SP

Figure 13. Matrix vesicles in the mature mantle predentin matrix.

In this electron micrograph, a view of the mature matrix is shown adjacent to the basal lamina and this is similar to the region outlined in figure 12 (at a higher magnification). Preodontoblast cell processes extend nearly to the basal lamina (BL) of the preameloblasts (PAm). Banded, collagen fibers (CO) are a prominent feature of the mature mantle predentin matrix and they extend to the basal lamina (BL). The matrix vesicles (arrowheads), stationed near the basal lamina, appear as electron opaque bodies. Alkaline phosphatase activity is demonstrated by the small, electron opaque granules in the matrix space (arrows). The reaction product appears associated with banded collagen fibers, cell processes, and the electron lucent space (possible ground substance). The reaction product appears also to be associated with the matrix vesicles. (37,500X)

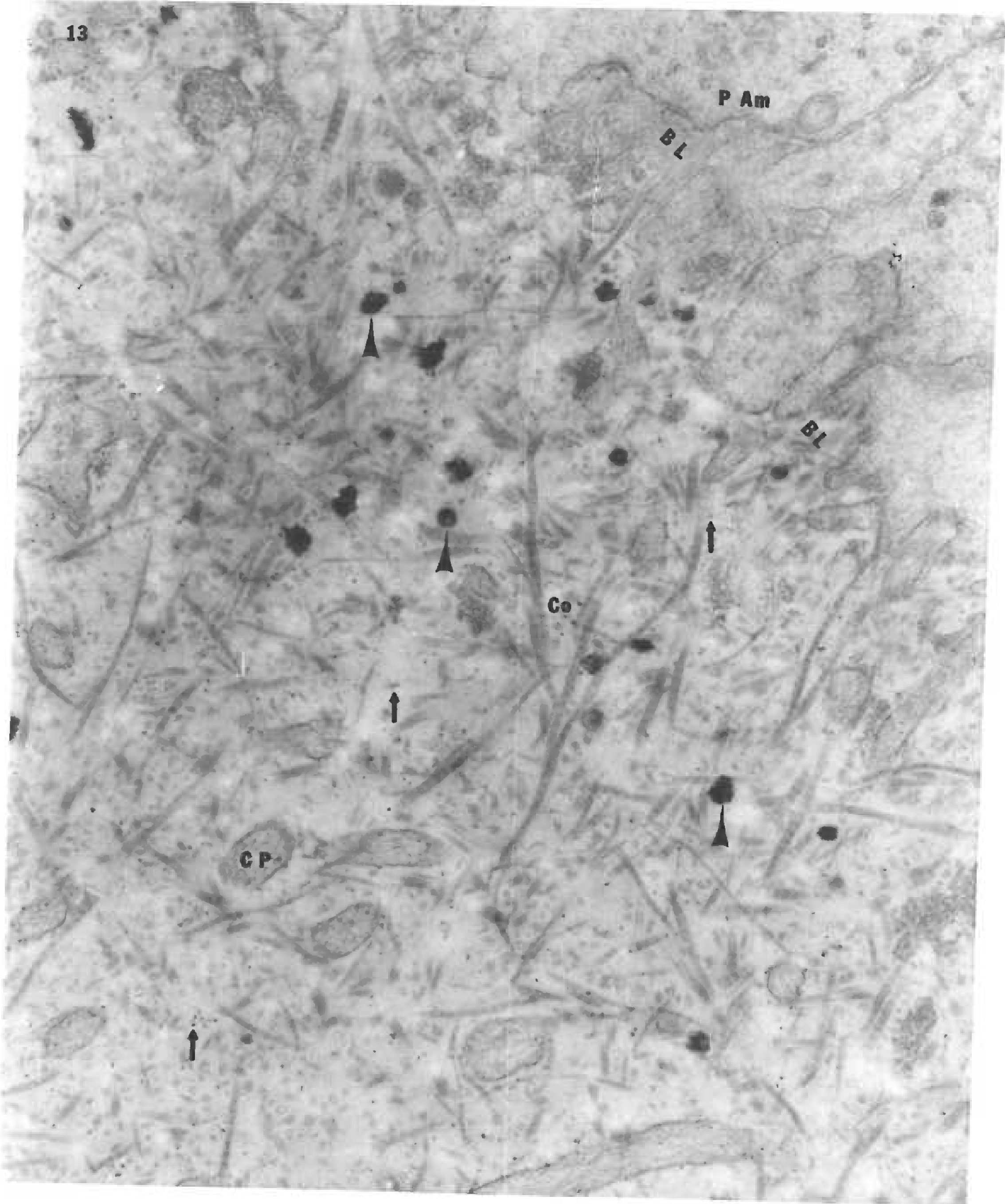
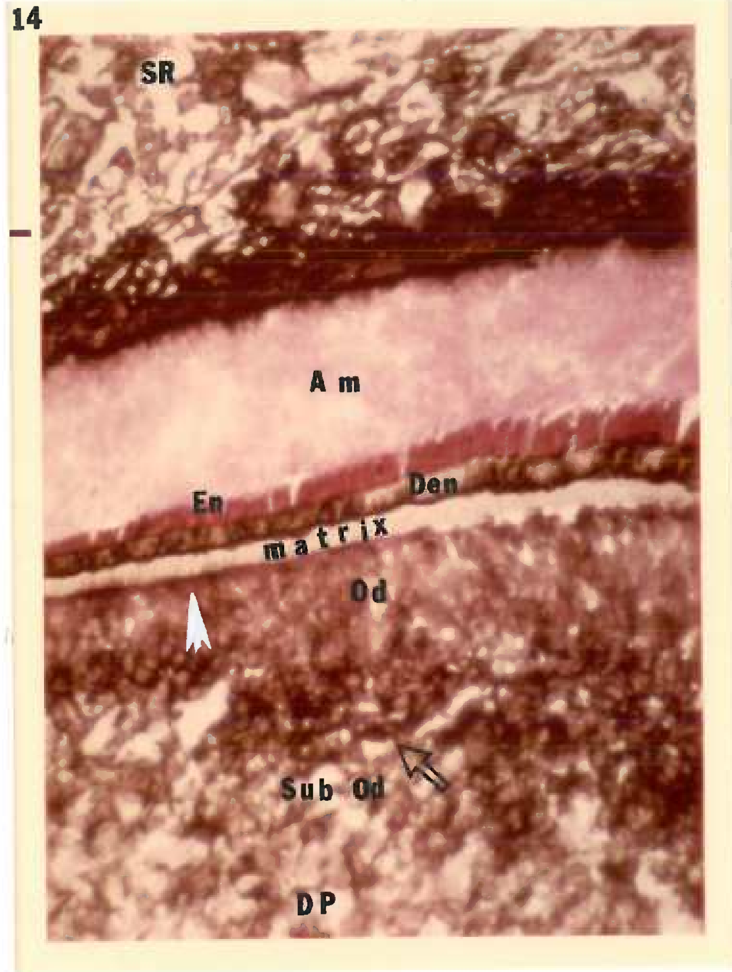

13
P Am
BL
BL
Co
CP

Figure 14. The histochemistry of the two day old rat first molar tooth germ: the cusp region in cross section.

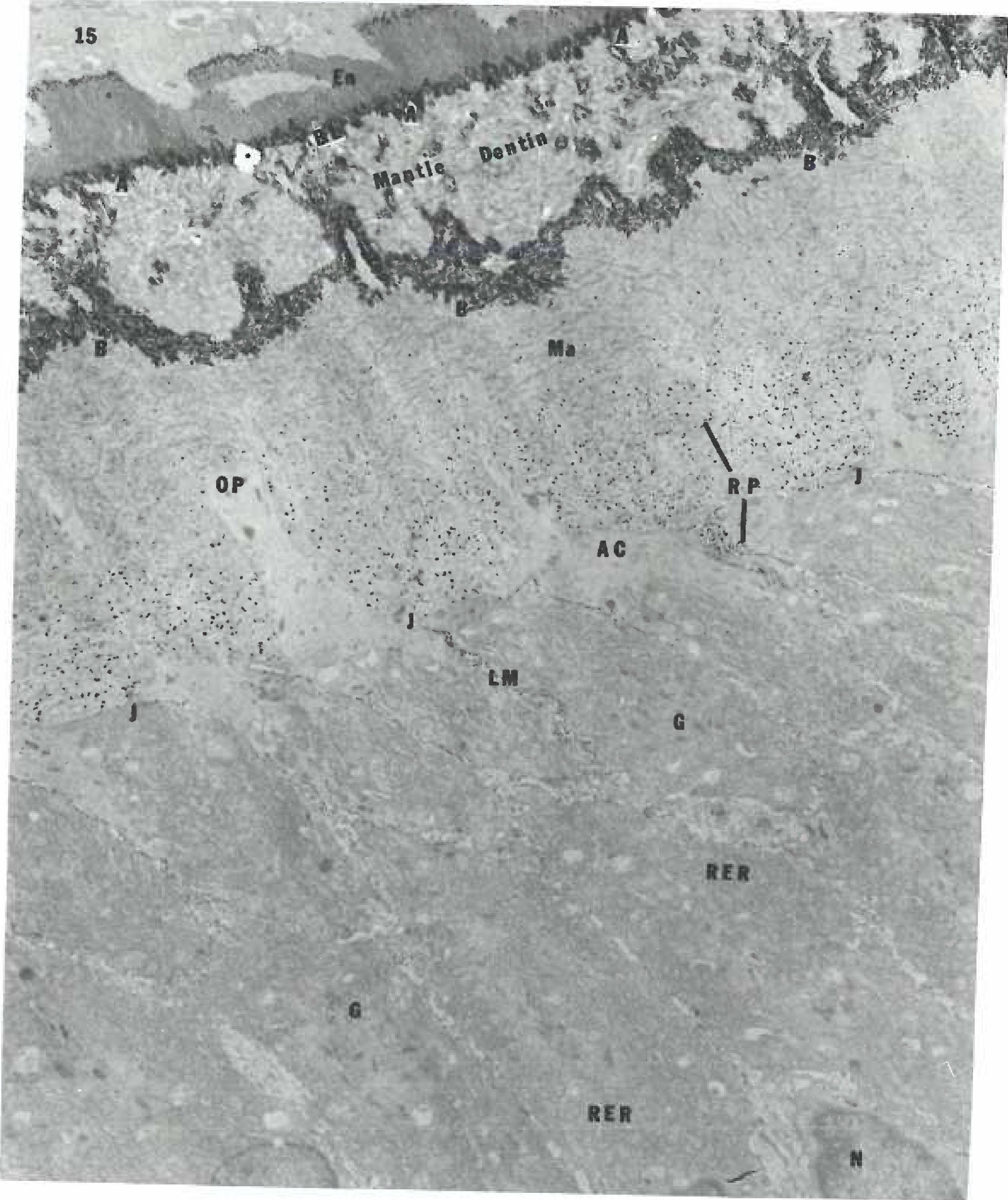
Both dentin (Den) and enamel (En) are evident in the cusp region of the two day old rat first molar. The dentin consists primarily of mantle dentin (Den), which formed in the mature mantle predentin matrix space. Adjacent to the mantle dentin, lies the circumpulpal predentin matrix. Alkaline phosphatase activity, black reaction product, is shown concentrated in the stratum intermedium (SI) layer, but lacking in the ameloblast (Am) layer. A moderate deposit is evident at the predentin matrix-odontoblast interface (arrowheads) and moderate to heavy deposit is evident in the subodontoblast region (open arrows). Alkaline phosphatase; substrate beta glycerol-phosphate, counter stain nuclear fast red, modified Gomori lead salt technique. (96X)



14
SR
A m
En
Den
matrix
Od
Sub Od
DP

Figure 15. Overview of enamel, mantle dentin, circumpulpal predentin matrix and the mature, secretory odontoblast.

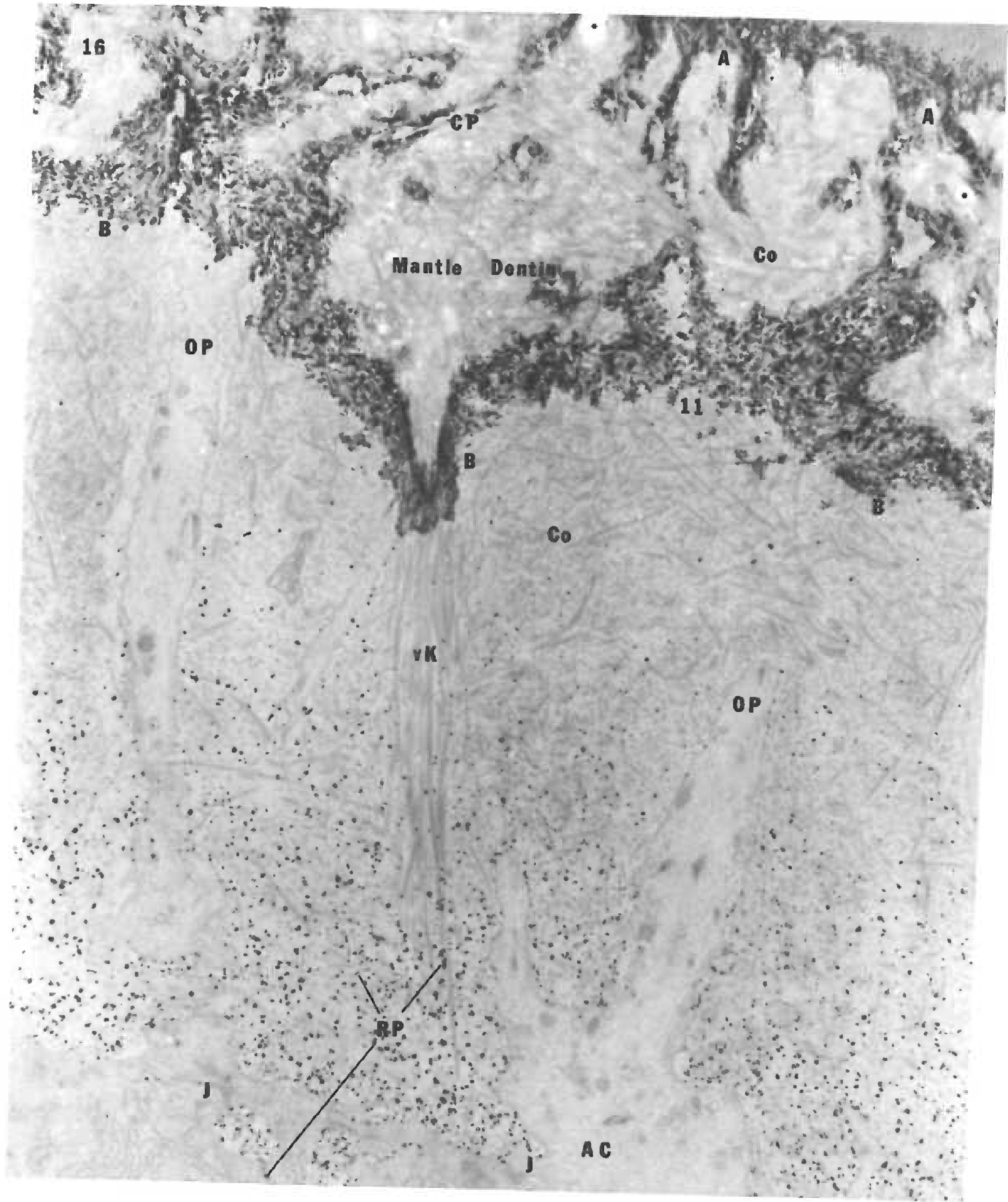
This electron micrograph demonstrates the cusp region of the two day old rat first molar. Mantle dentin is demarcated by two parallel ribbons of calcification (A and B). Calcification ribbon A is in the area previously occupied by the fibers of the basal lamina. Calcification ribbon B represents the calcification front for circumpulpal dentin formation. Within the mantle dentin, electron opaque material appears to occur along the cell processes. The circumpulpal predentin matrix (Ma) is densely packed with collagen fibers and odontoblast cell processes (OP) extend into the mantle dentin. The odontoblasts appear tangentially sectioned with their basal nucleus and apical cytoplasm (AC) adjacent to the predentin matrix. The supranuclear cytoplasm lies between the apical cytoplasm and the basal nucleus. Alkaline phosphatase activity is evidenced by the reaction product in a zone which parallels the apical end of the odontoblast in the region of the newest formed collagen. Reaction product also appears along the lateral membrane (LM) of the odontoblast and reaction product is included in the apical intercellular junctions (J). (6,500X)



15
En
A
A
BL
Mantle Dentin
B
A
B
B
Ma
OP
RP
J
AC
J
LM
J
G
RER
G
RER
N

Figure 16. Mantle dentin and the circumpulpal predentin matrix.

Mantle dentin is shown sequestered between the two ribbons of calcification, A and B, while the circumpulpal predentin matrix lies between the calcification ribbon B and the apical end (AC) of the odontoblasts. The collagen (Co) of the remaining mantle predentin matrix appears indistinct when compared to the collagen in the circumpulpal predentin matrix. The electron opaque crystals appear along the odontoblast process (OP) membrane within the confines of the mantle dentin. A large bundle of fibers, von Korff fibers (vK), appear in the predentin matrix attached to the calcification front B. Alkaline phosphatase activity is shown by the reaction product to be in a zone adjacent to the apical end (AC) of the odontoblast in the region of the most recently deposited collagen (Co). This is contrasted with the apparent absence of reaction product in the predentin matrix adjacent to the calcification front B which represents the region of the most mature collagen (Co) fibers. Enzyme activity is also associated with the odontoblasts membrane. (14,250X)



16
CP
A
A
B
Mantle Dentin
Co
OP
11
B
B
Co
vK
OP
RP
J
J
AC

Figure 17. Circumpulpal predentin matrix.

The fine structure of the circumpulpal predentin matrix is shown from the apical cytoplasm (AC) of the odontoblast to the circumpulpal dentin calcification front B. The von Korff (vK) fibers appear to parallel the odontoblast process (OP), while the remainder of the collagen of the matrix appears primarily in cross section or perpendicular to the process. The banding of the circumpulpal calcification front at B is distinct and appears in concert with the banding of the von Korff fibers (open arrows). The fibers adjacent to the calcification front, the mature collagen fibers, are densely packed while those fibers adjacent to the odontoblasts, are somewhat less densely packed. Alkaline phosphatase activity appears associated with the most recently formed fibers. (27,500X)

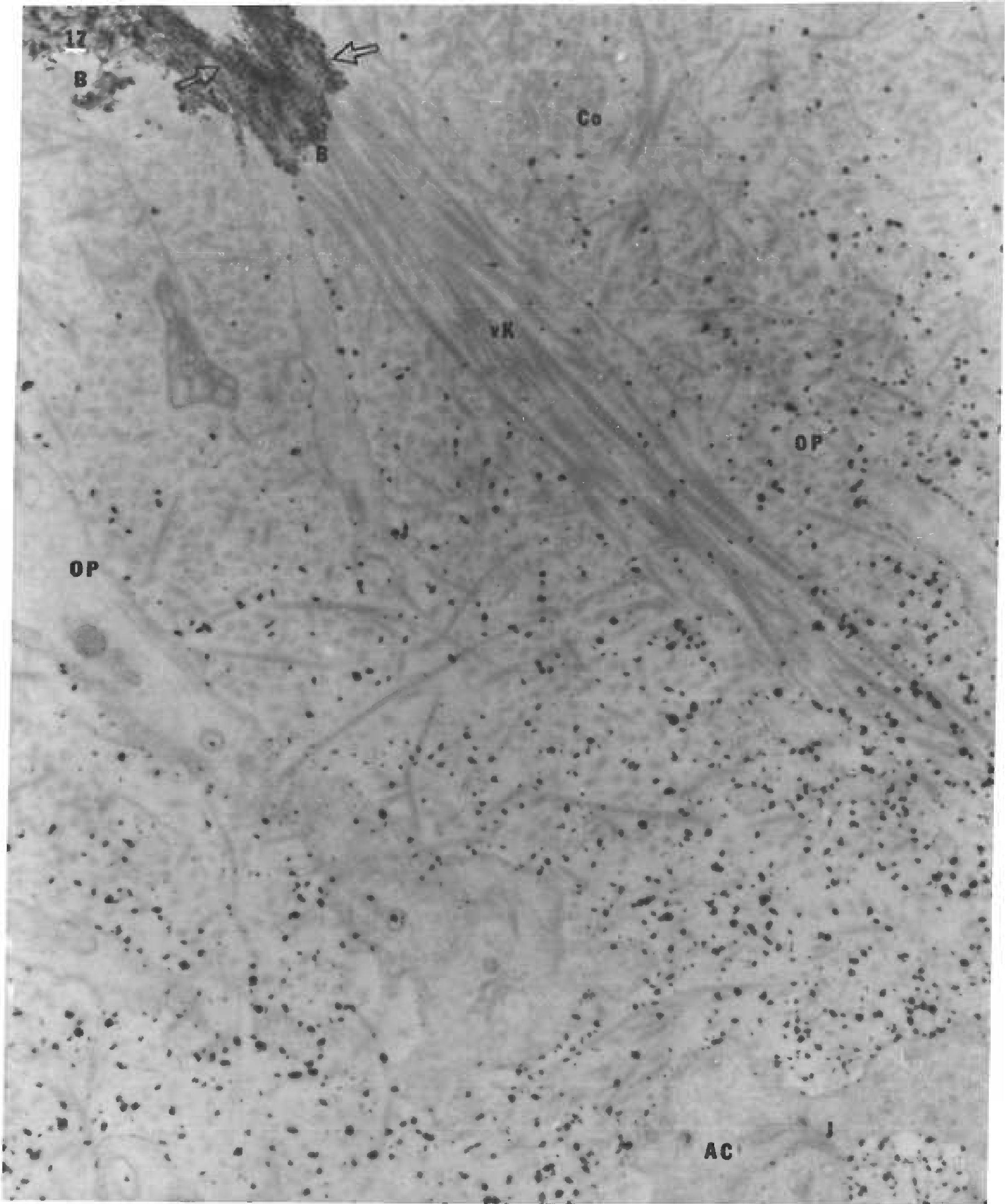
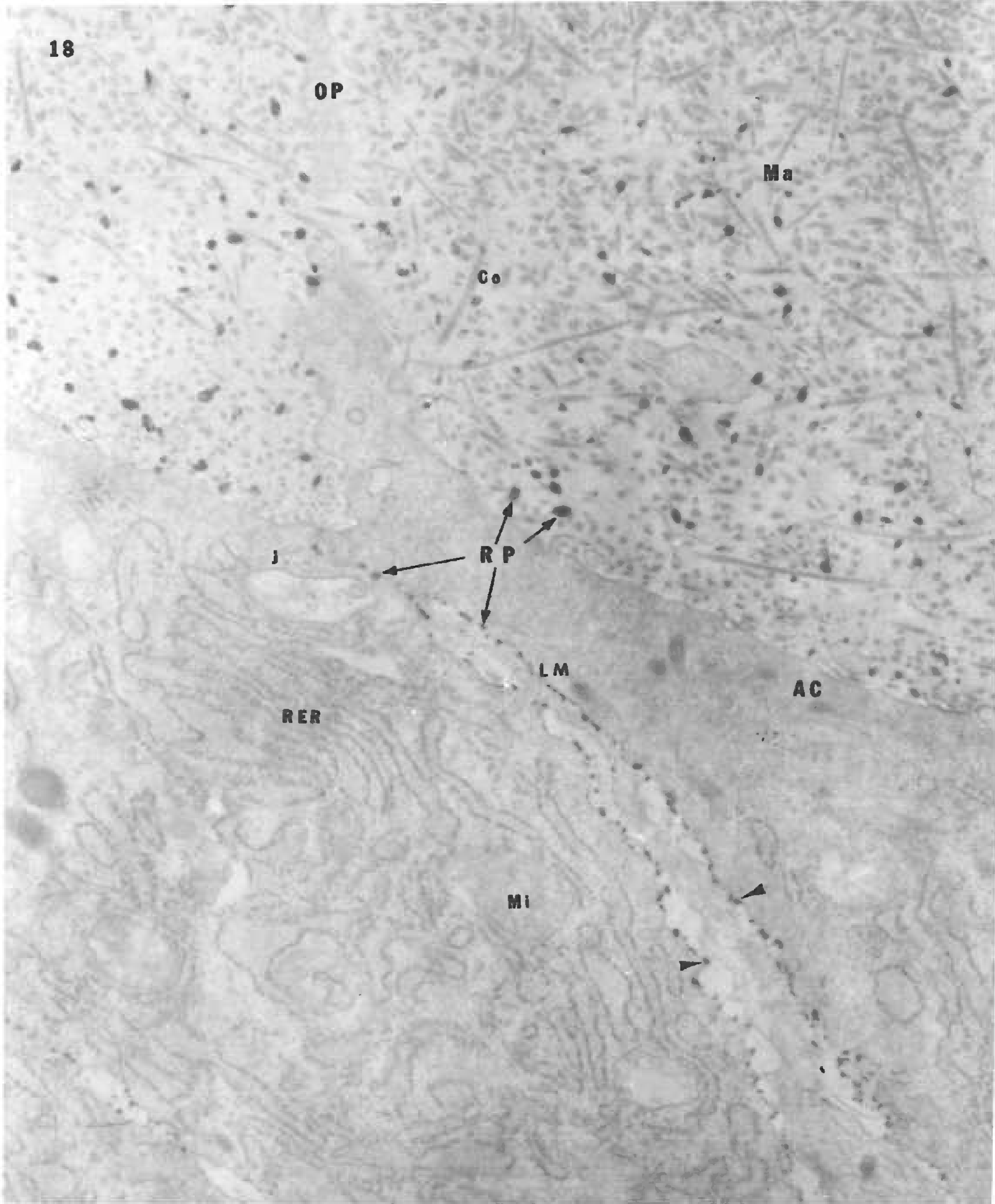

17
B
B
Co
vK
OP
OP
AC
J

Figure 18. The interface between the odontoblast and the circumpulpal predentin matrix,

Ultrastructual details of the odontoblast-circumpulpal predentin matrix interface are shown. In the extra cellular matrix, collagen fibers (Co) are banded and distinct, and they are frequently associated with oblong granular reaction product (arrows). Reaction product along the lateral odontoblast membrane (arrows) is distinctively smaller than the matrix reaction product and appears associated with the outer leaflet of the lateral membrane (arrowheads). (38,000X)



18
OP
Ma
Co
J
R P
LM
AC
RER
Mi

Figure 19. Substrate free control.

In the cusp region of the two day rat molar, banded collagen fibers (Co) are a prominent feature of the circumpulpal predentin matrix in this figure. Regions of apparent calcification, which appear as electron opaque crystalline-like structures, are demonstrated. Alkaline phosphatase activity is not evident in the matrix adjacent to the odontoblast in this substrate free control specimen. (32,000X)

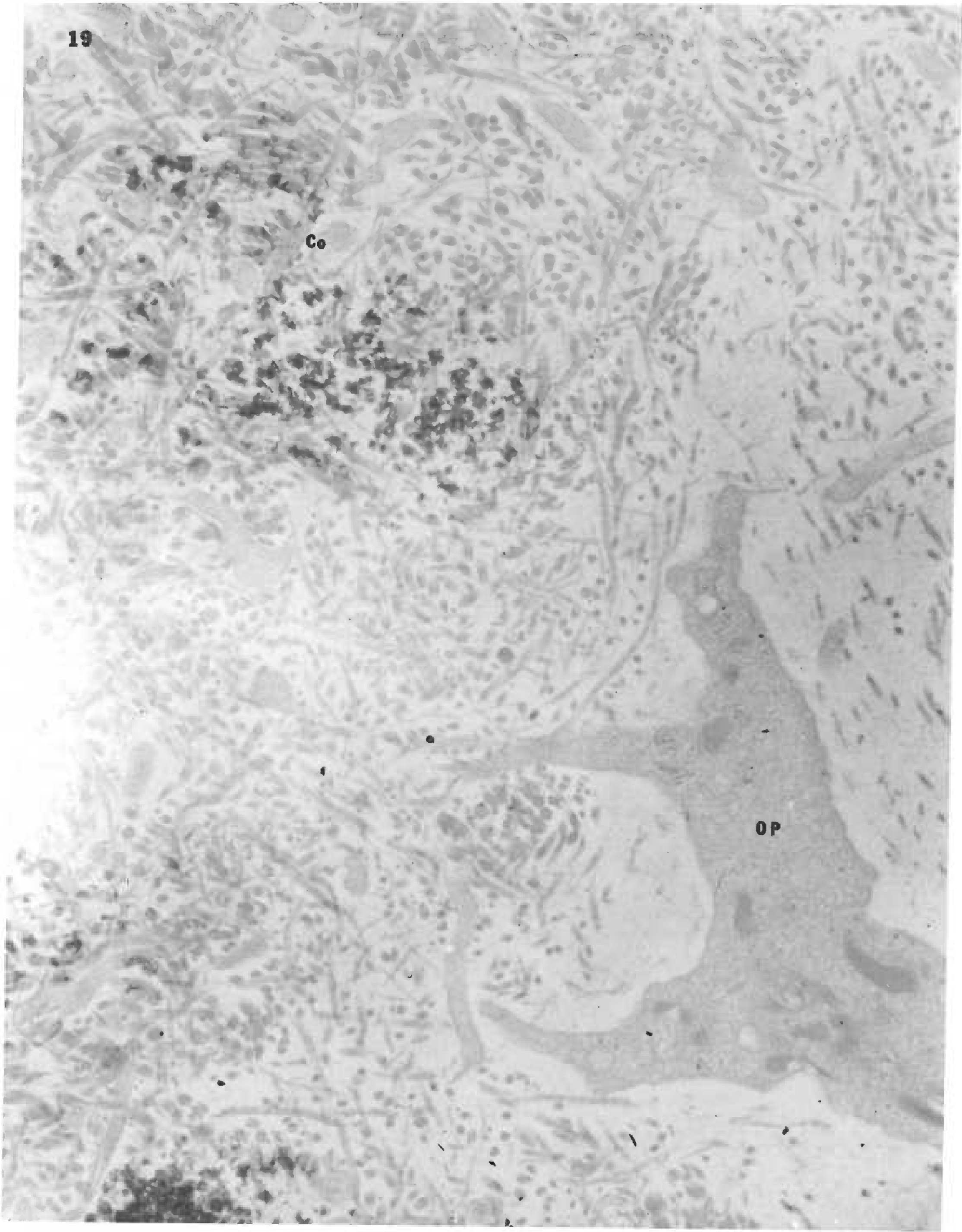

19
Co
OP

Figure 20. Cloudy control.

A region from the two day molar of the circumpulpal predentin matrix is shown near the apical end of the odontoblast. A generalized precipitate is dispersed in both intra- and extracellular regions. This is presumably not the result of enzyme activity since this cloudy control specimen was treated to deactivate or inhibit the activity of alkaline phosphatase prior to incubation. (47,500X)

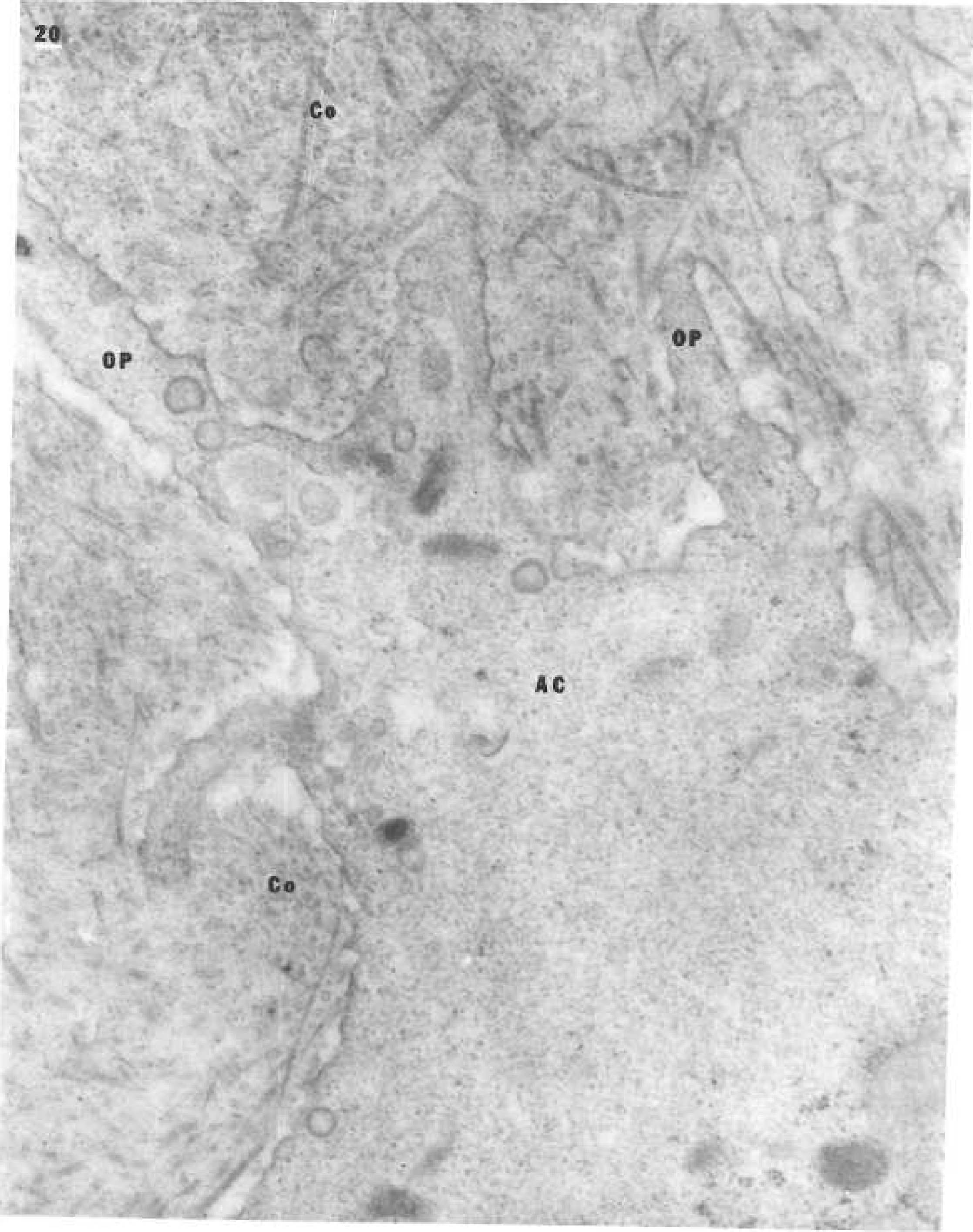

20
Co
OP
OP
AC
Co

Figure 21. Histology of the cusp region of the two day rat first molar.

First molar tooth germ tissue relationships are shown in the light micrograph in this two day old rat pup specimen. The odontoblasts form an epithelial-like border adjacent to the predentin matrix. Mantle dentin is evident between the dark staining enamel and the circumpulpal predentin matrix. Histologic preparation. (140X)

Figure 22. The secretory odontoblasts and the circumpulpal predentin matrix.

Odontoblasts, sectioned tangentially, are shown with the adjacent predentin matrix and the calcified dentin. The odontoblasts are polarized with a basal nucleus (N) and an apical cytoplasmic process (OP) which penetrates the matrix (Ma). Golgi complexes (G) are evident as are extensive arrays of rough endoplasmic reticulum (RER). Numerous secretory packages (SP) are demonstrated in the Golgi and appear regularly from the Golgi to the apical cytoplasm (AC). The predentin matrix (Ma) appears densely populated with collagen fibers. Alkaline phosphatase activity is membrane associated along the lateral odontoblast membrane (LM) and extracellular in the matrix (Ma). The matrix reaction product appears to exist as a zone adjacent to the odontoblasts and this corresponds to the most recently formed collagen. In contrast, reaction product is not apparent adjacent to the calcified dentin, which corresponds to the mature collagen prior to calcification. (6,500X)

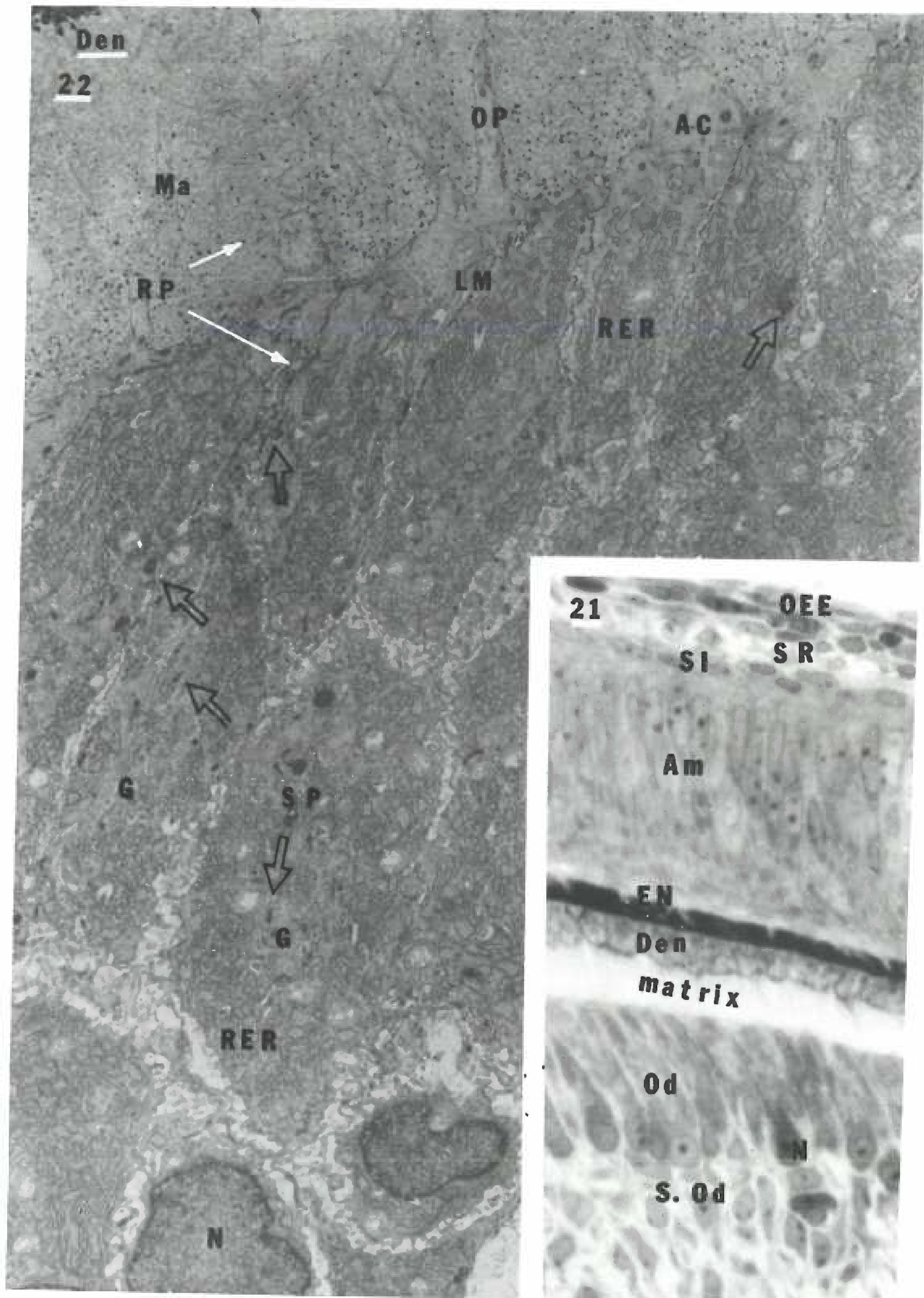

Den
22
OP
AC
Ma
RP
LM
RER
G
SP
G
RER
N
21
OEE
SI
SR
Am
EN
Den
matrix
Od
N
S. Od

Figure 23. Procollagen in the Golgi complex.

The Golgi region located near the lateral membrane of a secreting odontoblast is shown. Within the Golgi, an elongated fibrous structure (procollagen) with periodic electron opaque particles (EOP) is shown with other Golgi elements (Sac). While a membrane generally encloses the apparent collagen precursors in the Golgi, the membranes in this figure are not distinct. Alkaline phosphatase reaction product (RP) is evident along the lateral membrane (LM). (85,000X)

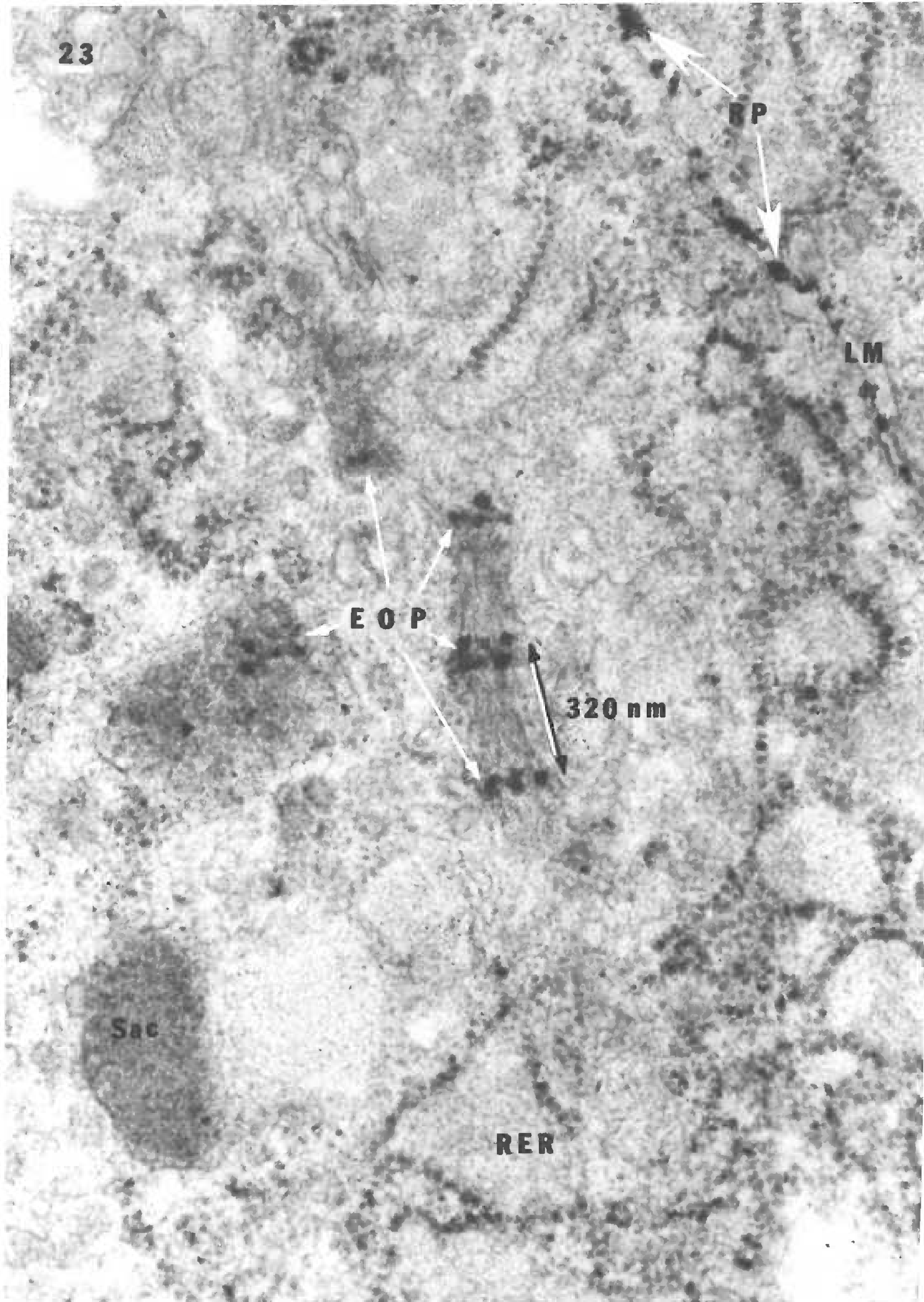
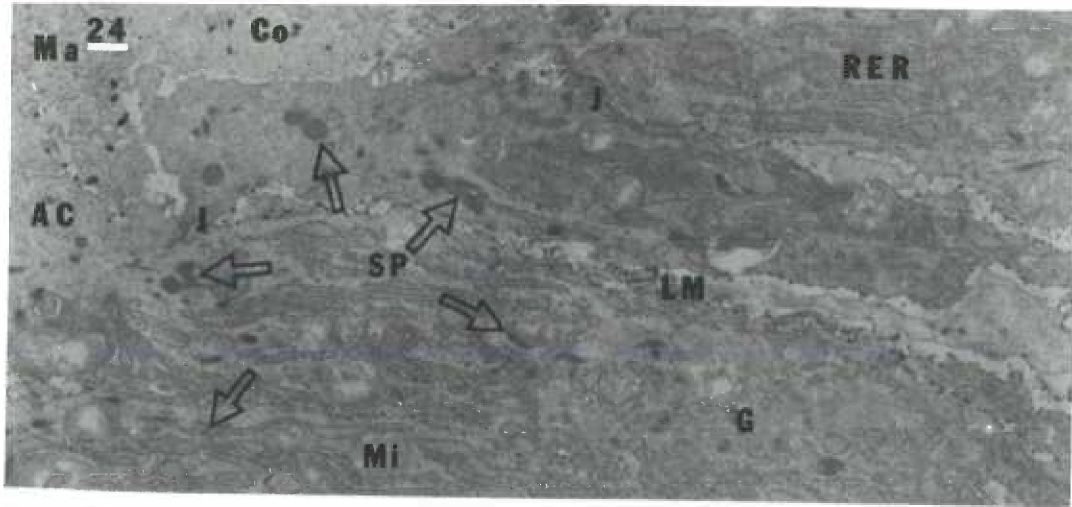

23
RP
LM
EOP
320 nm
Sac
RER

Figure 24. Secretory packages in the Golgi and apical cytoplasm, and adjacent predentin matrix.

This low power electron micrograph illustrates a portion of several odontoblasts, including the Golgi region, and the apical cytoplasm (AC), and the adjacent circumpulpal predentin matrix. Secretory packages are noticeable in the Golgi, along the lateral membrane (LM), and in the apical cytoplasm (AC) of a single odontoblast. Alkaline phosphatase activity appears along the lateral membrane (LM) and in the predentin matrix among the collagen fibers. (22,750X)

Figure 25. Synthetic cytoplasm and apical cytoplasm of the odontoblast and the predentin matrix.

The transition region between the organelle laden cytoplasm (synthetic cytoplasm) and the apical cytoplasm (AC) of the odontoblast is shown. Secretory packages (SP), exhibiting a variable morphology (open arrows), are evident in both regions of the cytoplasm. Alkaline phosphatase activity appears along the lateral membrane (LM) and among the collagen fiber cross sections (Co) in the matrix (Ma). (47,500X)


Ma
24
Co
RER
J
AC
J
SP
LM
G
Mi

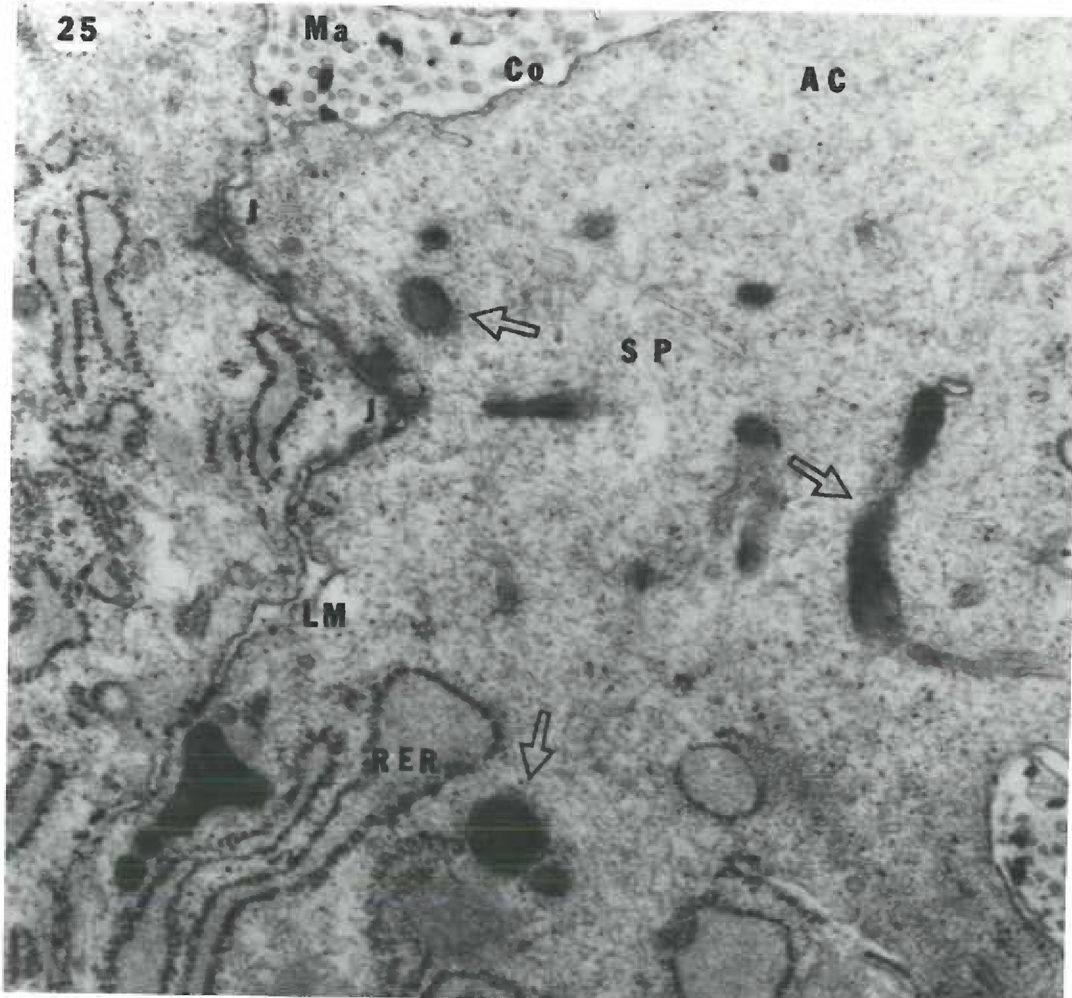

25
Ma
Co
AC
J
SP
J
LM
RER

Figure 26. Secretory packages.

Secretory packages are shown in the apical cytoplasm of the secreting odontoblasts. Two longitudinal, tubular packages appear membrane limited, with fine, longitudinal fibrills and periodic electron opaque particles (EOP). The packages in cross section exhibit a fine punctate appearance. The electron opaque particles (EOP) of a package are located at the periphery, but within the limiting membrane. The particles appear at approximately ninety degree intervals around the perimeter of the package. (227,500X)

Figure 27. Secretory package.

An elongated, tubular secretory package is shown with electron opaque particles. The limiting membrane and fibrillar contents appear continuous through the length of the package. (177,500X)

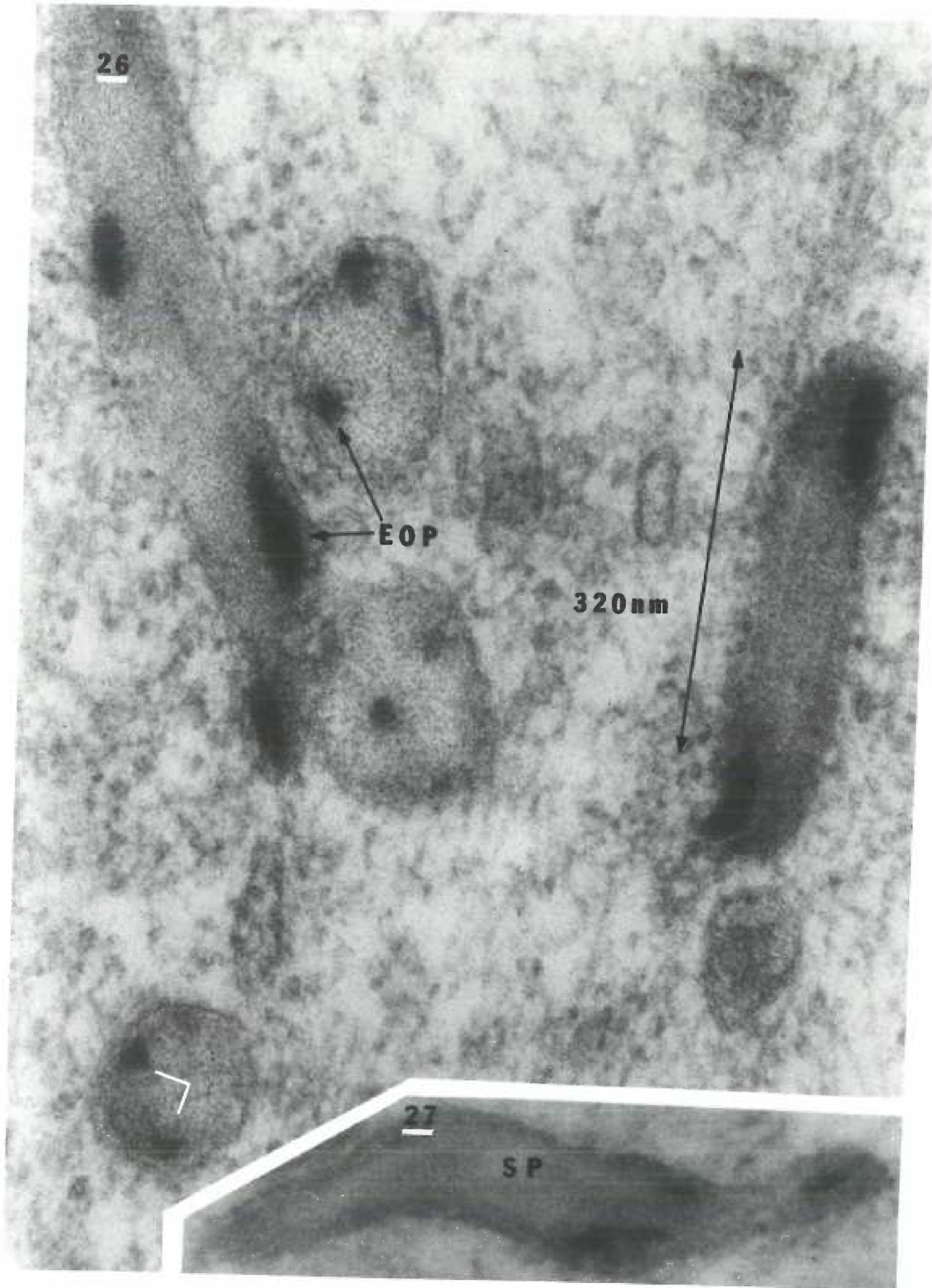

26
EOP
320nm
27
SP

Figure 28. Secretory package and attached vesicle.

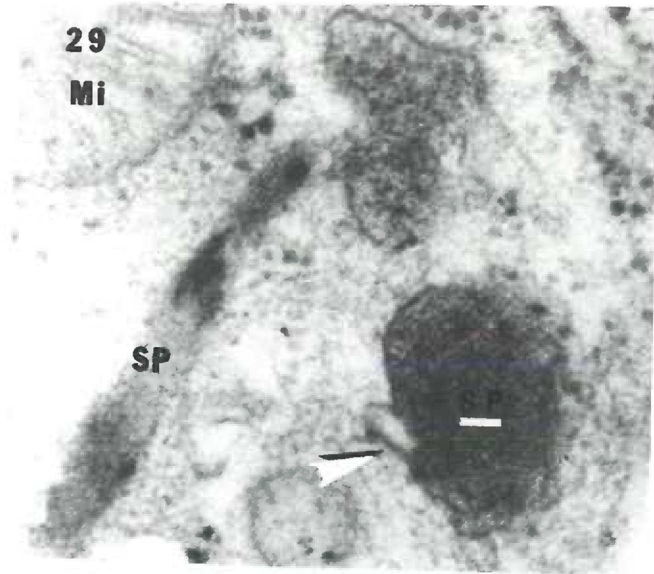
A membrane bound secretory package (SP) located in the lateral cytoplasm near the junction is shown among cytoplasmic elements including a mitochondrion (Mi). Alkaline phosphatase activity (RP) is associated with the lateral membrane (LM) of the odontoblast. A vesicle (V) with a protrusion (arrowhead) appears intimately associated with the secretory package. (142,500X)

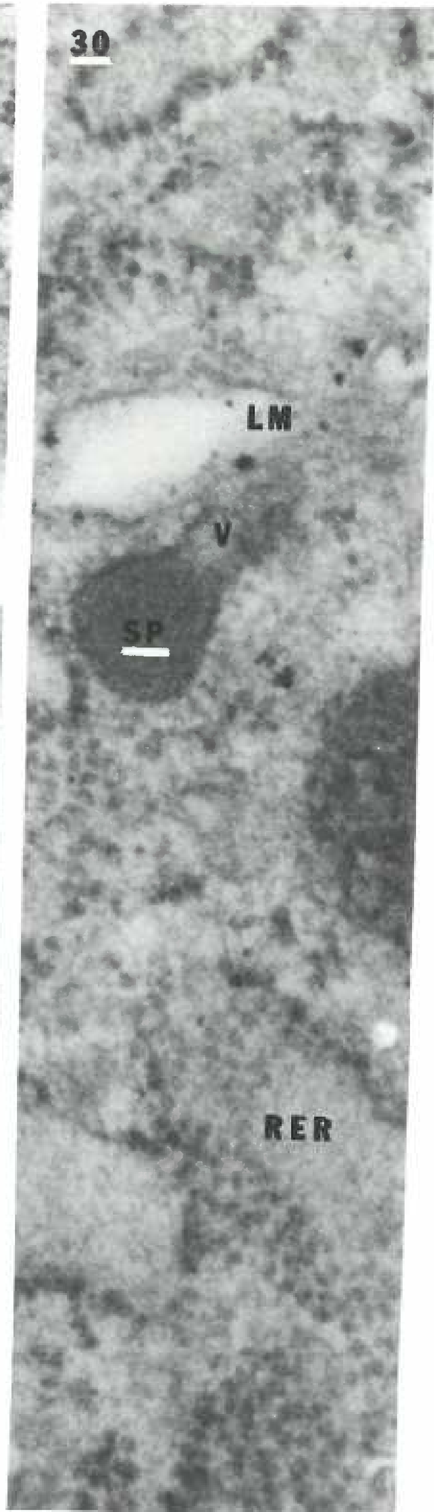
Figure 29. Secretory package and apparent attached protrusion.

A secretory package situated similarly in the odontoblast to the one in figure 28 is shown. An attached protrusion, presumably from a vesicle, is continuous with the membrane of the package (arrowhead). (142,500X)

Figure 30. Secretory package - vesicle "blister formation".

A secretory package (SP) with a distinct membrane is shown near the lateral odontoblast membrane (LM) and the associated reaction product (RP). A continuous membrane encloses the secretory package (SP) and an attached vesicle (V), which appears as an electron lucent "blister." The contents of the vesicle (V) and package (SP) appear confluent. (142,500X)


29
Mi
SP
SP


30
LM
V
SP
RER

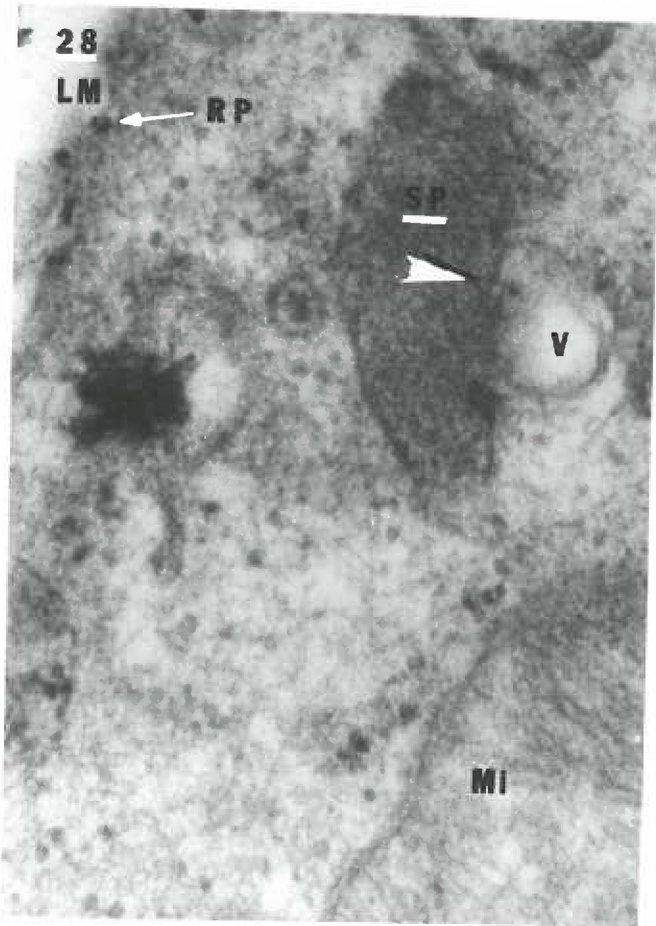
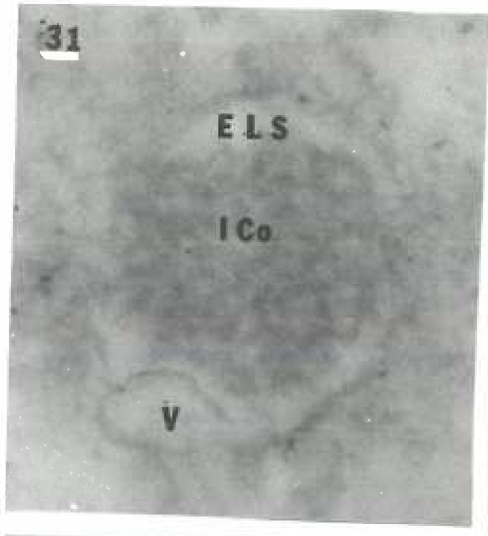

28
LM
RP
SP
V
Mi

Figure 31. Intracellular intermediate collagen.

A secretory package with an attached vesicle (V) is shown. An electron lucent space (ELS) nearly isolates the package contents (ICo) from the membrane, and the space is continuous with the vesicle (V). Coincident with the development of the electron lucent space (ELS), the terminology of secretory package (SP) converts to intermediate collagen (ICo). The apparent absence of the electron opaque particles is characteristic of the conversion from the secretory package to the intermediate collagen (ICo). The contents are no longer fibrillar but instead exhibit an irregular fibrous appearance which will be defined as intermediate collagen. (142,500X)

Figure 32. Intracellular intermediate collagen.

Two secretory elements are shown near the lateral membrane of the odontoblast just below the apical cytoplasm. The secretory package (SP) exhibits minor membrane irregularities, but it appears as a typical intracellular package. In contrast, the intermediate collagen exhibits an electron lucent space (ELS) which completely isolates the irregular fibrous structure from the limiting membrane. Note that neither the intracellular, intermediate collagen within the electron lucent space nor the limiting membrane of the space contain alkaline phosphatase activity. (142,500X)


31
ELS
ICo
V

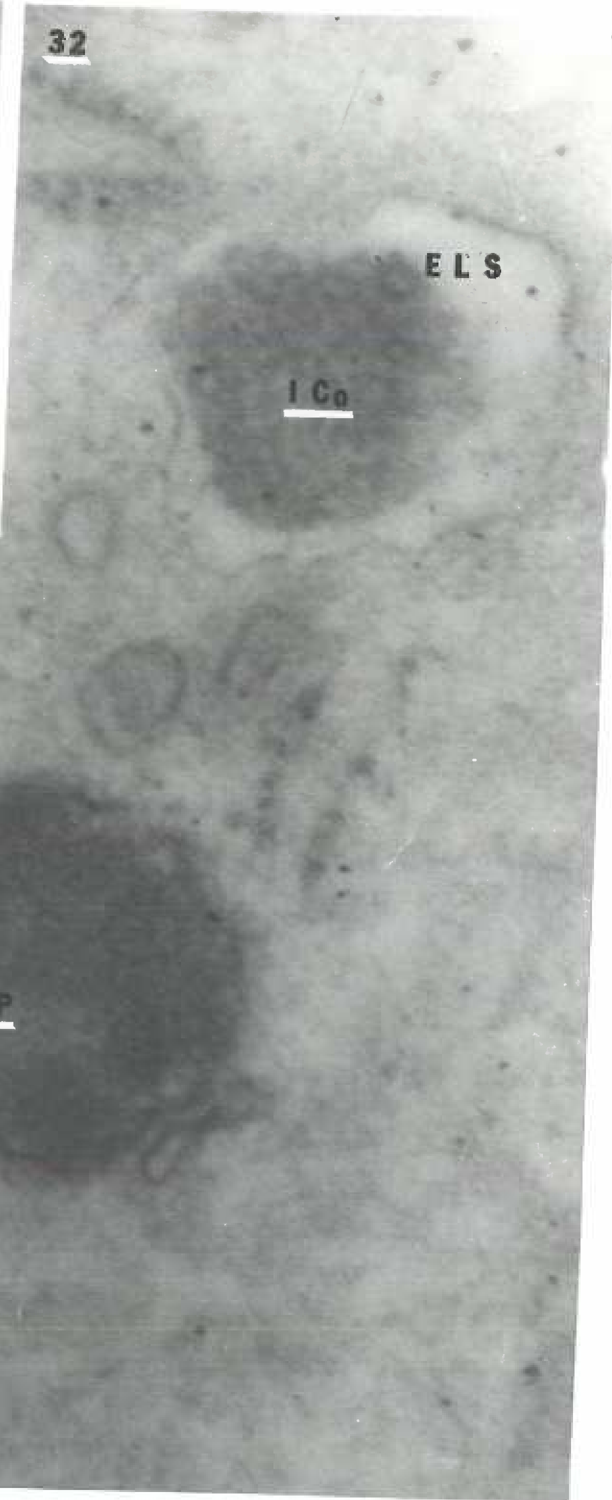
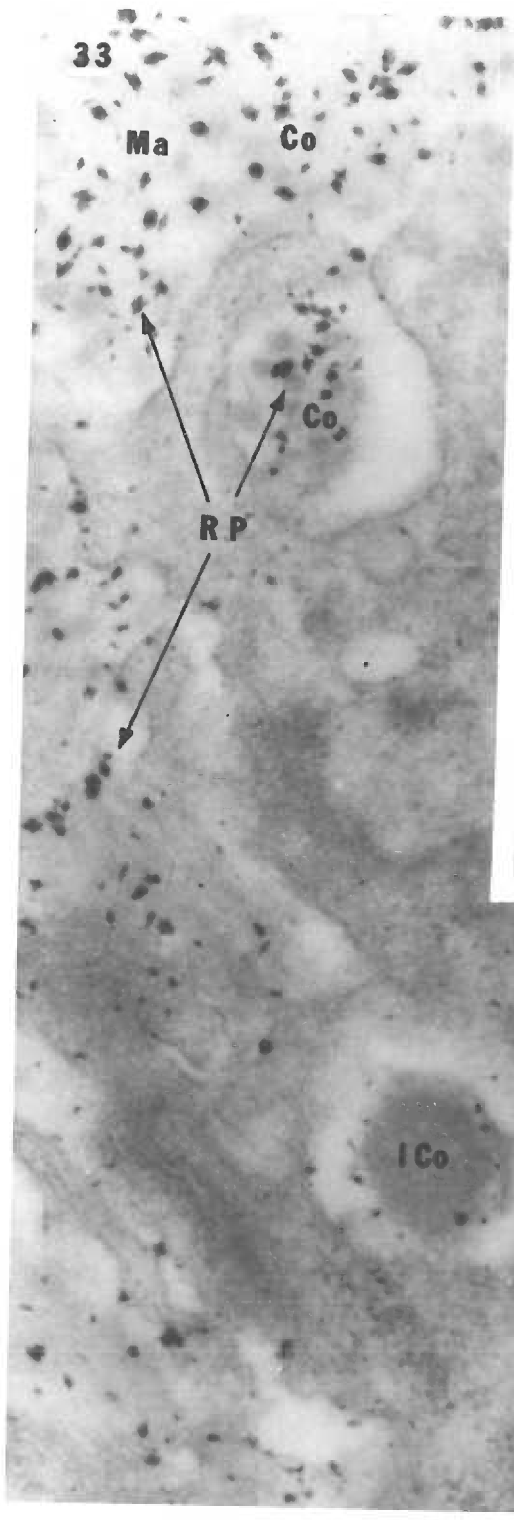

32
ELS
ICo
SP

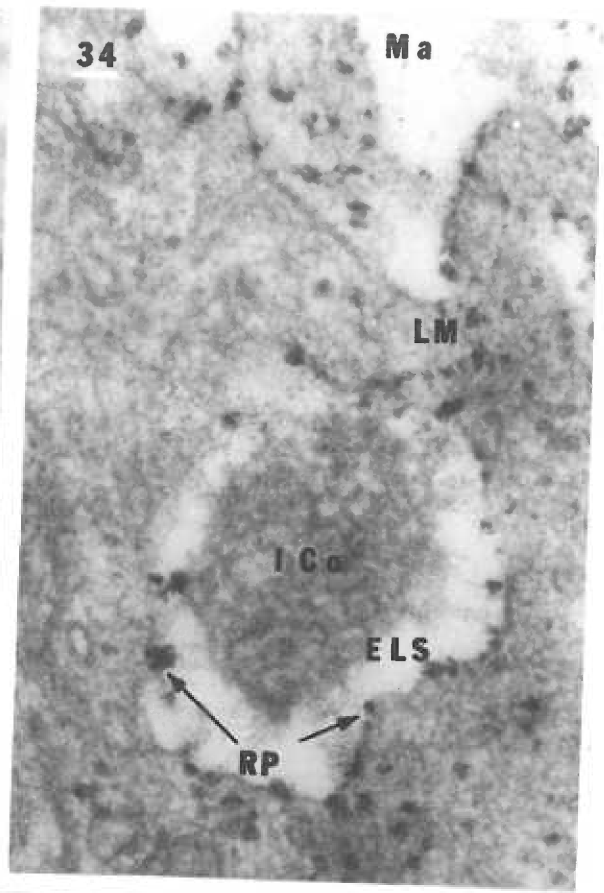
Figure 33. Extracellular intermediate collagen.

Intermediate collagen (ICo) with an electron lucent space (ELS) is shown between adjacent odontoblasts along the lateral membrane (LM). Alkaline phosphatase activity (RP), not previously associated with the intracellular packages, now appears at the periphery of the intermediate collagen (ICo) as well as along the lateral odontoblast membrane. Reaction product is also noted in the predentin matrix (Ma) associated with the collagen fibers (Co). (85,000X)

Figure 34. Extracellular intermediate collagen.

The intermediate collagen (ICo) is isolated by a continuous electron lucent space (ELS), with thin filamentous attachments between the intermediate collagen (ICo) and the lateral odontoblast or limiting membrane. Alkaline phosphatase reaction product (RP) appears associated with the limiting membrane surrounding the intermediate collagen (ICo). In addition, reaction product is observed at the points of attachment of the fine filaments that bridge the electron lucent space (ELS) and connect the intermediate collagen (ICo) with the lateral limiting membrane (LM). (142,500X)


33
Ma
Co
Co
RP


34
Ma
LM
ICo
ELS
RP

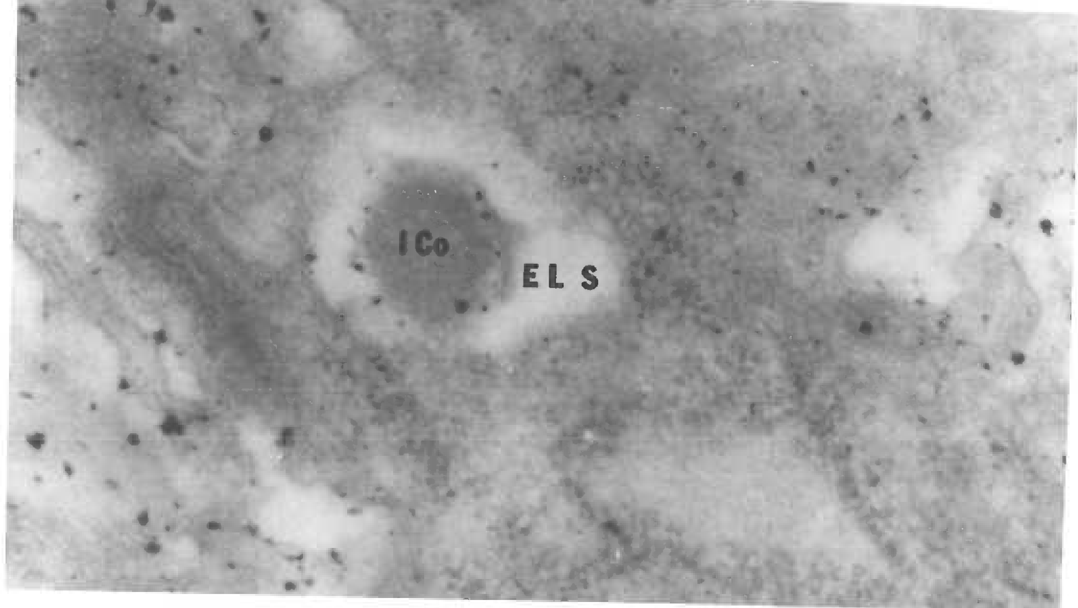
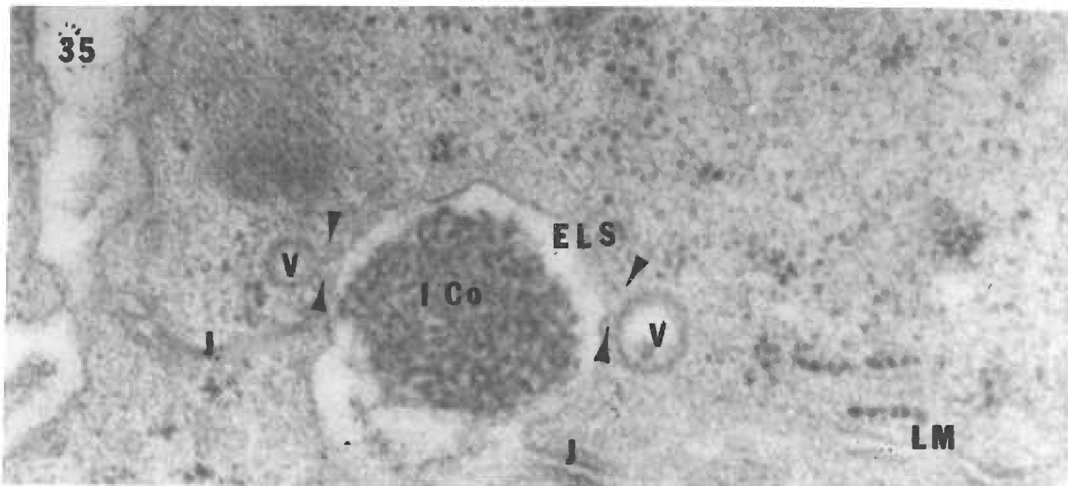

ICo
ELS

Figure 35. Cloudy control of the extracellular intermediate collagen.

In this cloudy control preparation, the intermediate collagen (ICo) is shown lying between two adjacent odontoblasts. Note the position of the intermediate collagen (ICo) relative to the cell junctions (J). An electron lucent space (ELS) isolates the intermediate collagen (ICO) from the limiting membrane, and this membrane is shown to be continuous with the junction membrane and lateral membrane of the odontoblasts. Two vesicles (V) are attached to the intermediate collagen membrane (arrowheads) and their contents appear confluent with the electron lucent space (ELS). Alkaline phosphatase activity is not a feature of this control preparation. (112,500X)

Figure 36. Transport of collagen.

Portions of three odontoblasts are shown demonstrating lateral membranes and associated reaction product (RP). Three secretory packages a, b, and c, demonstrate the sequential deposition of collagen. It appears as an intracellular secretory package, a, as an intracellular collagen intermediate, b, and as an extracellular collagen intermediate, c. Note the alkaline phosphatase activity associated with the extracellular collagen (ICo) but not with the intracellular collagen (SP). Enzyme activity is evident along the lateral odontoblast membrane (LM). (61,500X)


35
ELS
V
ICo
V
J
J
LM

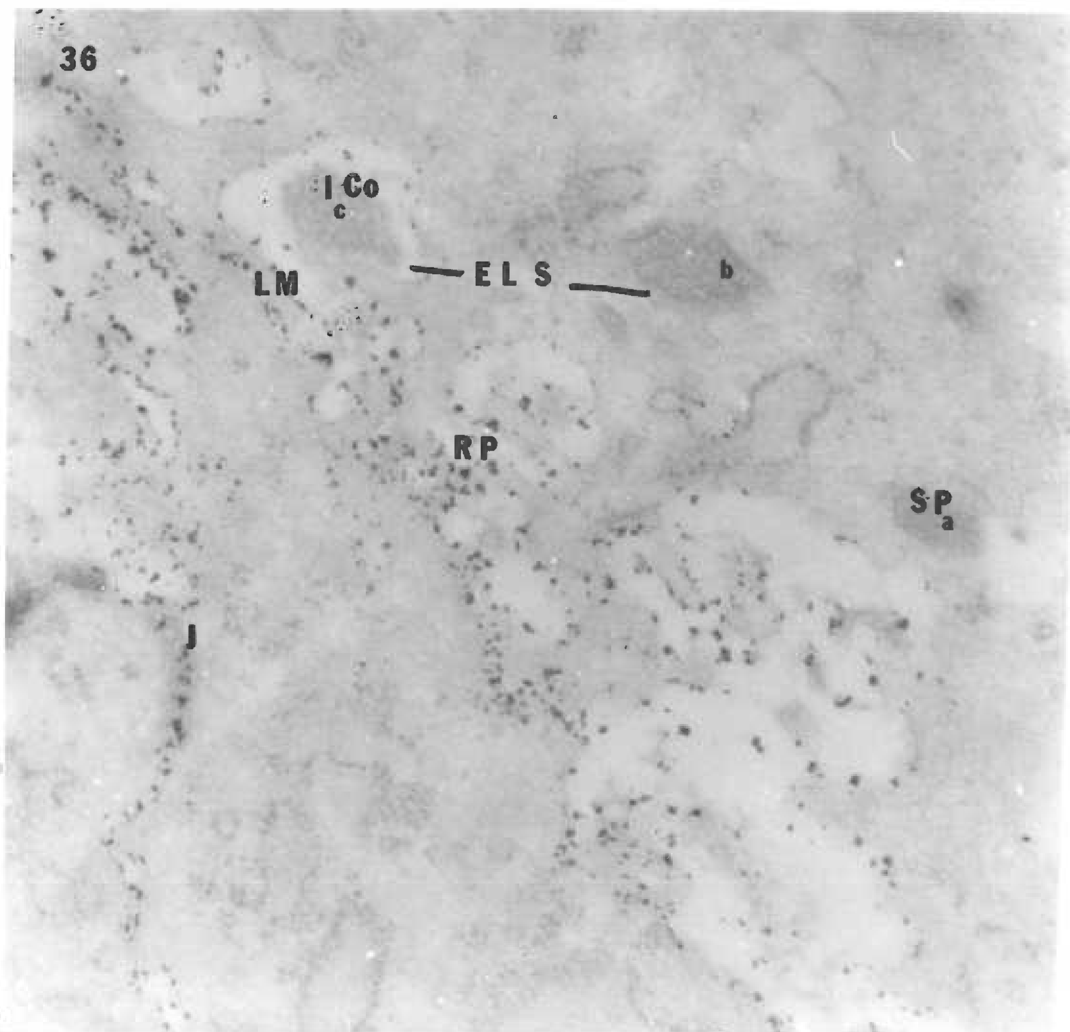
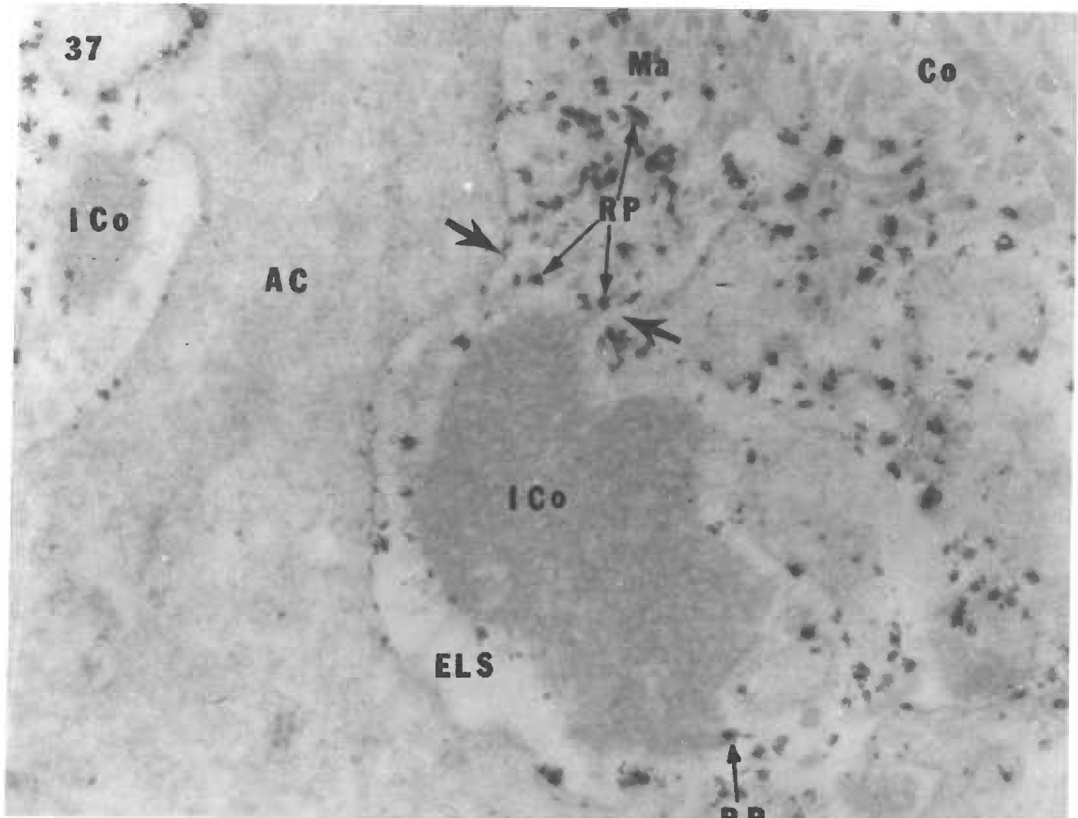

36
ICo
c
LM
ELS
b
RP
SP
a
J

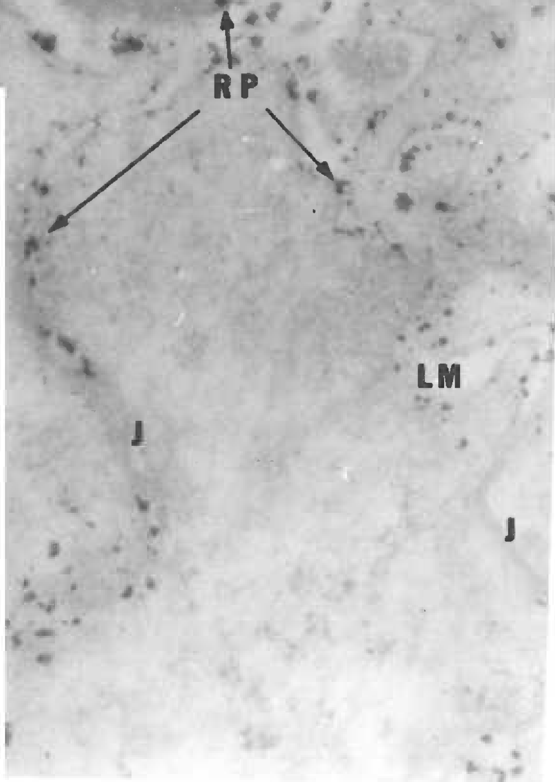
Figure 37. Extracellular intermediate collagen: exposure to the predentin matrix.

Intermediate collagen (ICo) with direct exposure to the predentin matrix (between the thick arrowheads), is shown between adjacent odontoblasts. The morphological outline of the intermediate collagen (ICo) appears threaded or screw shaped or helical and it is isolated by an electron lucent space (ELS) from the odontoblast. Fibers emanate directly from the intermediate collagen into the matrix at the region of exposure (arrowheads). Alkaline phosphatase activity (RP) is localized on the periphery of the collagen intermediate and on the odontoblast membrane adjacent to the electron lucent space (ELS). Reaction product also appears associated with collagen fibers in the predentin matrix. (47,500X)

Figure 38. Extracellular intermediate collagen: cloudy control

In this cloudy control preparation, intermediate collagen exhibits a morphological outline similar to the intermediate collagen shown in figure 37. An electron lucent space (ELS) isolates the intermediate collagen (ICo) from the adjacent odontoblast apical cytoplasm (AC). A generalized, granular deposit is contrasted with the typical reaction product of alkaline phosophatase activity. (47,500X)


37
Ma
Co
I Co
RP
AC
I Co
ELS


RP
LM
J
J

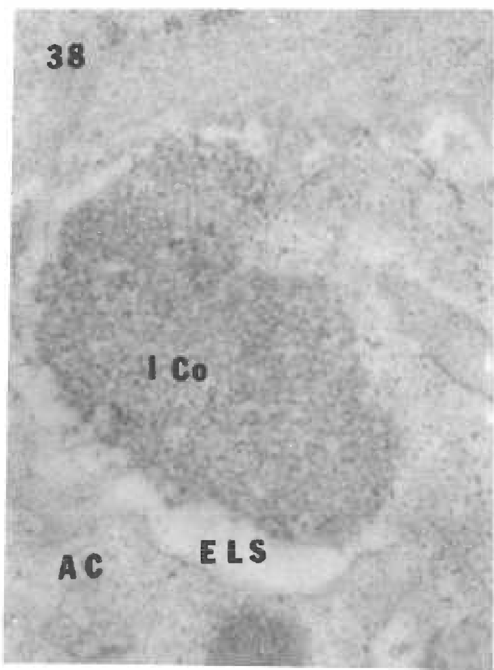
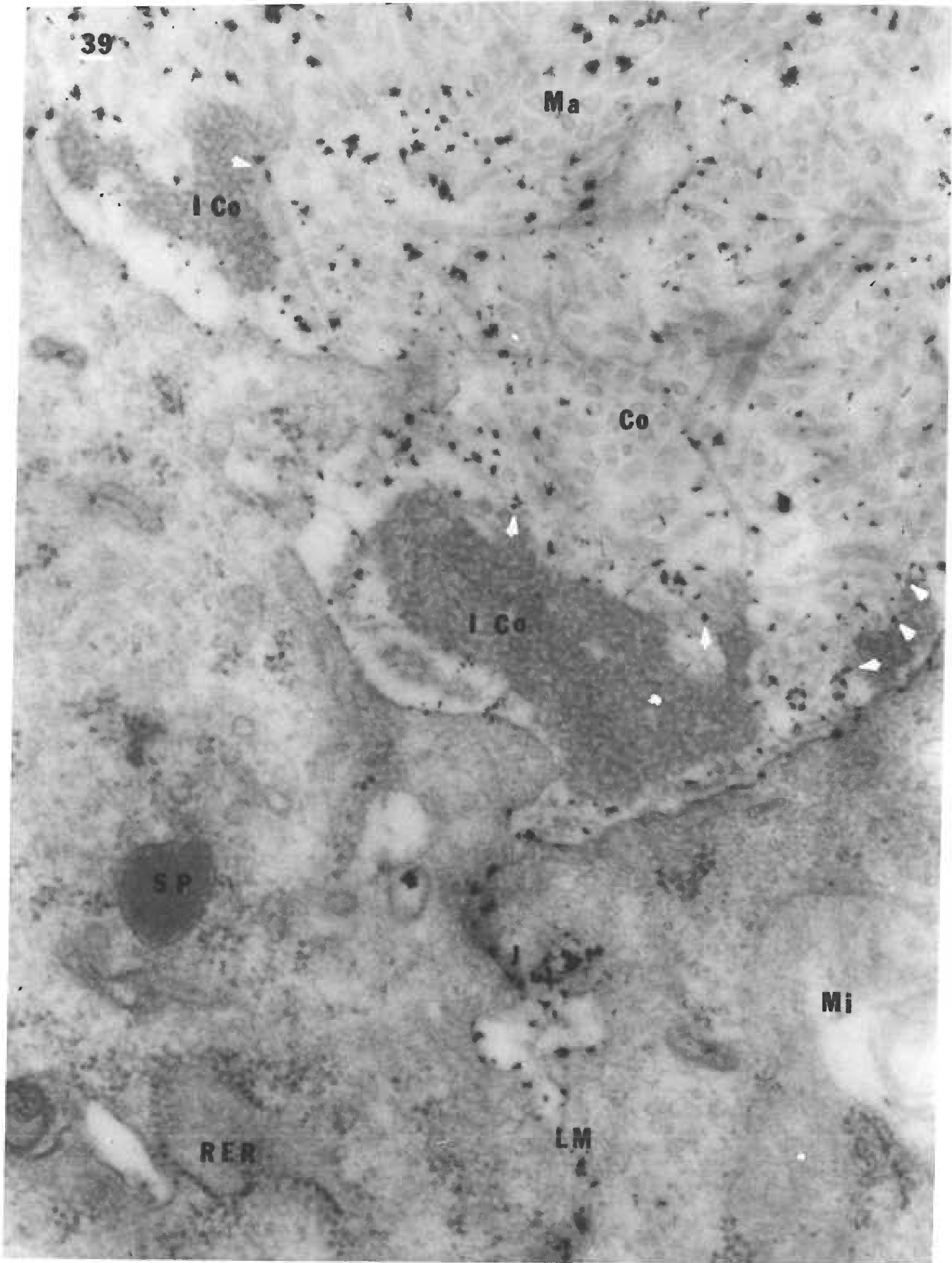

38
I Co
AC
ELS

Figure 39. Intermediate collagen: origin of banded collagen fibers.

Intermediate collagen (ICo) is shown between adjacent odontoblasts and it exhibits an extensive exposure to the predentin matrix (Ma). An electron lucent space separates the intermediate collagen (ICo) from the apical odontoblast membrane while banded collagen fibers emanate directly into the matrix from the intermediate (arrowheads). Alkaline phosphatase reaction product appears at the transition point of the intermediate collagen with the banded collagen fibers (arrowheads). Enzyme activity also appears in the predentin matrix associated with the collagen fibers, and appears along the lateral membranes (LM) of adjacent odontoblasts. (85,000X)


39
Ma
I Co
Co
I Co
SP
J
Mi
RER
LM