THE EFFECT OF ADRIAMYCIN ON THE ALKALINE PHOSPHATASE ACTIVITY OF RAT INCISOR PULP

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ABSTRACT

Cytochemical techniques were used to study the effect of Adriamycin on the alkaline phosphatase activity of rat incisor pulp cells. Young male Sprague-Dawley rats (10 days old) were injected subcutaneously with Adriamycin (5.0 mg/kg) and killed by perfusion with a 2% buffered formaldehyde solution at 3, 5 and 7 days after injection. The pulps were either dissected out prior to demineralization, or were used after the incisors were demineralized in an EDTA solution. Incubation of the pulp segments was carried out in one of 4 reaction mediums for increasing periods of time. Electron microscopic observations revealed osteodentin formation in the Adriamycin treated groups. Associated with the osteodentin were odontoblast-like cells. The latter exhibited a higher alkaline phosphatase activity than untreated pulp cells. This activity was seen primarily in association with the plasma membranes, and along the secretory pathway of the cells. This activity increased with longer incubation times. In the EDTA-treated incisors the reactions were greatly reduced or were absent. The results indicated that Adriamycin caused the differentiation of pulp cells into odontoblast-like cells that were associated with an osteodentin matrix. These cells showed a high alkaline phosphatase activity, indicating that they may be involved in the mineralization of the osteodentin matrix through the

transport of metabolites. This process appears to be associated (and probably occurs simultaneously) with protein secretion.

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LIST OF ABBREVIATIONS

Adriamycin- trade name for doxorubicin hydrochloride; trade-mark property of Adria Laboratories of Canada, Ltd.

APase- non-specific alkaline phosphatase (E.C. 3.1.3.1)

ATP- adenosine triphosphate

DDSA- dodecenyl succinic anhydride

DMP-30- dimethyl amino methyl phenol

EDTA- disodium ethylenediaminetetraacetate

EGTA- ethylene glycol bis-(B-aminoethyl ether) N,N,N',N'-tetracetic acid

ER- endoplasmic reticulum

Mg^{2+}-,Ca^{2+}-ATPase- magnesium-, calcium-activated adenosine triphosphatase (E.C. 3.6.1.3)

MVBs- multivesicular bodies

Na^{+}-,K^{+}-ATPase- sodium-, potassium-activated adenosine triphosphatase (E.C. 3.6.1.3)

NMA- nadic methyl anhydride

Pi- inorganic phosphate

PPi- inorganic pyrophosphate

SO zone- subodontoblastic zone

UA- uranyl acetate

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INTRODUCTION

Alkaline Phosphatase

Research concerning enzymes capable of hydrolyzing phosphate esters extends back over 75 years. Suzuki, Yoshimura and Takaishi in 1907 reported the existence of an enzyme in cereals that acted upon phytin to release inorganic phosphate. This was confirmed the next year by Harden and Young (1908), who also found that the optimal activity of this enzyme was in an alkaline medium. Subsequent research showed that extracts of mammalian tissues, particularly kidney and intestinal mucosa, contained enzymes that hydrolyzed hexose phosphates and glycerophosphates (Grosser and Husler 1912; Von Euler 1912).

The year 1923 saw the beginning of a period of intense investigation into phosphatases after the discovery that ossifying cartilage was a rich source of these enzymes (Robison 1923). Robison and his group discovered many aspects of this new enzyme, such as the proposed role of phosphatase in the mineralization of tissues (Robison 1923); the optimal pH for bone phosphatase, which was 8.4-9.4 (Robison and Soames 1924); and the optimal pH for phosphatase in soft tissues, such as kidney, intestinal mucosa and blood plasma, which was similar to that of bone phosphatase (Martland and Robison 1924). Other soft tissues with high phosphatase activity, such as liver and spleen, were found

to have an additional phosphatase activity at pH 5 (Davies 1934). It was further proposed by Davies that the terms acid and alkaline phosphatase be employed to distinguish between the enzymes. Also, many phosphate derivatives were found to be good substrates for these enzymes. These derivatives included hexose phosphates, glycerophosphates and ethyl, adenyl, and phenyl phosphates (Kay 1932). By 1936, it was discovered that many different classes of phosphatases were in existence, and some were substrate specific. As well, it was discovered that one tissue could contain several different types of phosphatases (Folley and Kay 1936).

The development of a histochemical method demonstrating alkaline phosphatase activity in tissues (Gomori 1939; Takamatsu 1939) was followed by extensive investigation into the localization of the enzyme, especially in mammalian tissues. Many species were employed, and a wide range of mineralizing tissues were investigated, such as bone (Gomori 1943; Burstone 1960; Jeffree 1962), calcifying cartilage (Gomori 1943; Morse and Greep 1951; Burstone 1960), and dental tissues (Engle and Furuta 1942; Gomori 1943; Harris 1950; Symons 1955b; Burstone 1960; Ten Cate 1962, 1966; Goggins and Fullmer 1967; Kiguel 1970). These enzymes have also been demonstrated in a number of soft tissues such as kidney, intestinal mucosa, liver, and placenta (for a review, see Roche 1950 and Fishman 1974).

With the introduction of the electron microscope, the ultrastructural localization of alkaline phosphatases within cells of various tissues was investigated. The presence of the enzymes in many soft tissues was confirmed, and enzyme activity was also detected in mineralized tissue-producing cells.

Alkaline phosphatase activity has been associated with dental tissues ever since a histochemical technique for its localization was developed (Gomori 1939, 1943). Hard tissue formation in the tooth is an excellent example of the mineralization process, especially since the cells involved can be easily isolated and, in the case of odontoblasts, dissected out for study (Linde 1972). Most, if not all studies have reported enzyme activity in the stratum intermedium, the stellate reticulum, the inner dental epithelium and the subodontoblastic pulp cell zone. However, the presence of the enzyme in odontoblasts, secretory ameloblasts and the external dental epithelium is debatable. Earlier researchers found no evidence of enzyme activity associated with odontoblasts (Engle and Furuta 1942; Ten Cate 1962; Kiguel 1965, 1970), but more recent reports show strong evidence for the existence of some enzymatic activity associated with these cells (Harris 1950; Ten Cate 1966; Kurahashi and Yoshiki 1972; Leonard and Provenza 1973; Magnusson and Linde 1974; Hasselgren et al 1978; Orams and Snibson 1982; Takano, Ozawa and Crenshaw 1986). This

situation is similar to that of secretory ameloblasts. Earlier researchers reported no activity (Burstone 1960; Ten Cate 1963; Kiguel 1965, 1970), but more recently, activity of the enzyme associated with these cells has been observed (Kurahashi and Yoshiki 1972; Magnusson et al 1974; Granström and Linde 1976; Orams 1981; Orams and Snibson 1982). In all cases, the activity was associated with the cell membranes. With regards to the odontoblasts, little or no activity was found on the cytoplasmic processes within the dentinal tubules. With regards to ameloblasts, most activity was found in the proximal part of the cell, near the stratum intermedium.

Recently, alkaline phosphatase was found associated with the cementum layer of the tooth, specifically the cellular cementum, another mineralizing tissue of the tooth (Granström 1982).

The identification of a second form of alkaline phosphatase, adenosine triphosphatase, a highly substrate-specific enzyme, in many cells of the tooth was followed by a period of intense investigation and several theories regarding the role of both enzymes in the mineralizaton process. This second enzyme, a magnesium-, calcium-activated adenosine triphosphatase (Mg^{2+}-,Ca^{2+}-ATPase, E.C. 3.6.1.3) was discovered by researchers using sensitive histochemical and biochemical techniques, and by employing selective inhibitors of the various alkaline phosphatases

found within the cells (Borgers 1973). Mg^{2+}-,Ca^{2+}-ATPase has been demonstrated in ameloblasts (both presecretory and secretory), odontoblasts, and the cells of the pulp, in particular the pulp cells of the subodontoblastic zone. Employing both light microscopic and electron microscopic techniques, the ultrastructural localization of this enzyme has been determined as well. Presecretory ameloblasts show activity on the cellular membranes, especially at the proximal end near the stratum intermedium (Severson 1968; Magnusson et al 1974; Magnusson and Linde 1974; Takano, Ozawa and Crenshaw 1986). The secretory ameloblasts, however, show activity over the entire cell membrane, especially the Tomes processes (Severson 1968; Crenshaw and Takano 1982; Takano, Ozawa and Crenshaw 1986; Sasaki and Garant 1986). Severson (1968) and Crenshaw and Takano (1982) noticed some activity on the maturation stage ameloblasts, but this was primarily limited to the base of the cells. Odontoblasts have been reported to exhibit Mg^{2+}-,Ca^{2+}-ATPase on the cellular membrane (Severson 1968; Magnusson and Linde 1974; Magnusson et al 1974) and intracellular vesicles (Granström, Linde and Nygren 1978; Linde and Granström 1978). Biochemical studies have also reported enzyme activity associated with the microsomal fractions of the odontoblasts (Jontell, Granström and Linde 1979; Granström and Linde 1981). Granström (1984) recently presented further evidence to support the localization of

the Mg^{2+}-,Ca^{2+}-ATPase in the smooth endoplasmic reticulum and Golgi body membrane fraction of odontoblasts. Pulp cells also exhibit some Mg^{2+}-,Ca^{2+}-ATPase activity, mostly on the cell membranes (Severson 1968; Magnusson et al 1974; Magnusson and Linde 1974; Guo and Messer 1976, 1978; Abiko 1977; LeBell 1981). It should be noted that in all studies concerning pulp cells, odontoblasts were included. The cells of the subodontoblastic layer were also included in the above studies, but histochemical analysis has demonstrated high Mg^{2+}-,Ca^{2+}-ATPase activity associated with the membranes of these cells. It is also interesting to note that blood vessels found in the dental tissues also exhibited some Mg^{2+}-,Ca^{2+}-ATPase activity (Goggins and Fullmer 1967; Severson 1968; Magnusson and Linde 1974; Crenshaw and Takano 1982).

The biochemical characterization and the localization of the alkaline phosphatases in the cells of the tooth have revealed some possible functions for these enzymes in the mineralization process. Robison, in 1923, was the first to put forward a theory concerning the relationship of the enzymes to mineralized tissues. He stated that the mineralization of the tissue would occur spontaneously if the concentration of calcium phosphate in the extracellular tissue fluid exceeded its saturation point. The phosphate was contributed by the action of the alkaline phosphatases upon organic phosphate esters. This was the generally

accepted function of the enzyme in all hard tissues, but with its discovery in non-mineralizing tissues, other possible functions for alkaline phosphatases were presented. Bevelander and Johnson (1945) thought that the enzyme activity was associated with cell proliferation and division. This idea was shared by Symons (1955a) and Kiguel (1970). A connection between fibrillogenesis and alkaline phosphatase activity was made by many researchers (Jeener 1947; Bradfield 1949, 1950; Kiguel 1965; Yoshiki and Kurahashi 1971). Ten Cate (1966) was more specific by associating the enzyme activity of certain dental cells with the synthesis of collagen fibres. Still another function associated with the alkaline phosphatase activity was the production of mucopolysaccharides (glycosaminoglycans) and other components of organic matrices associated with the enzyme-active cells (Ten Cate 1962, 1963; Kiguel 1964, 1970).

One theory that is widely held and bears some consideration is that put forward by Neuman and his group. They discovered that inorganic pyrophosphate (PPi) and adenosine triphosphate (ATP), both substrates for alkaline phosphatase, inhibited mineralization of tissues (Fleisch and Neuman 1961; Fleisch 1964). This evidence suggests the enzyme is responsible for splitting these and other phosphate esters, thus keeping these inhibitors from the areas of mineralization (Neuman and Neuman 1958; Fleisch and

Neuman 1960, 1961). While this theory has been supported by many authors, nothing conclusive has been presented thus far.

Alkaline phosphatase has also been associated with cells involved in the transport of various substances across cell membranes. This has especially been seen in odontoblasts, known for their transport of certain ions and substances from the pulpal matrix to the dentinal matrix. It has been postulated that the alkaline phosphatase located on the cell membranes may be selective in the substrates that are transported from the pulp core to be employed in either matrix formation or mineralization of the matrix (Burstone 1960; Ten Cate 1962, 1966; Kiguel 1970; Yoshiki and Kurahashi 1971).

With the discovery and subsequent characterization of Mg^{2+}-,Ca^{2+}-ATPase in odontoblasts and cells of the pulp, a new function relating this enzyme to the mineralization process was postulated. Anderson (1973) was the first to propose an ATP-energized "calcium pump" that could be responsible for the accumulation of calcium within extracellular matrix vesicles involved in tissue mineralization. This was supported by Ali and Evans (1973) who found that the formation of hydroxyapatite from matrix vesicles in calcifying cartilage was dependent upon the presence of calcium and ATP, and the reaction was enhanced in alkaline conditions. Guo and Messer (1976) first associated the

Mg^{2+}-,Ca^{2+}-ATPase of pulp cells with this possible role in relation to the mineralization of dentin. Abiko (1977) also mentioned that the Mg^{2+}-,Ca^{2+}-ATPase of dental pulp may promote calcium ion transport.

Linde and Granström, localizing Mg^{2+}-,Ca^{2+}-ATPase to the intracellular vesicles of odontoblasts, set out to prove the existence of a Ca^{2+}-pump in odontoblasts that may be involved in the mineralization of dentin. In a series of papers (Linde and Granström 1978; Granström and Linde 1981; Granström 1984), they showed that Ca^{2+} uptake by a microsomal fraction from odontoblasts, containing the intracellular vesicles in question, was dependent upon ATP being present. This uptake was attributed to the Mg^{2+}-,Ca^{2+}-ATPase and, to a lesser degree, non-specific alkaline phosphatase (APase, E.C. 3.1.3.1). As well, Granström believes that this Mg^{2+}-,Ca^{2+}-ATPase may be acting as a Ca^{2+}-pump within the odontoblasts for two reasons: first, to maintain calcium homeostasis within the cell and secondly, to assist in the extracellular mineralization of the dentin matrix. The latter may be accomplished through the formation of matrix vesicles incorporating this enzyme, especially in the formation of mantle dentin (Granström 1984).

Other authors have voiced similar theories involving Mg^{2+}-,Ca^{2+}-ATPases. LeBell (1981) thought that they played a similar role, that of intercellular ion transport, in the

cells of the human pulp. Crenshaw and Takano (1982) also associated the Mg^{2+}-,Ca^{2+}-ATPase activity of secretory ameloblasts with the transport of calcium ions into the matrix of the enamel. This view was shared by Reith (1983) who, in a highly quoted paper, felt that alkaline phosphatases in general were involved in calcium transport. Recently, Takano et al (1986) presented additional evidence of the Mg^{2+}-,Ca^{2+}-ATPase acting as a calcium pump involved with the matrix vesicle-mediated mineralization of dentin.

Dental Pulp

The dental pulp is a unique tissue. It is unlike any other tissue in the body in that it is enclosed within a rigid cavity of calcified tissue to which it contributes in its formation. The pulp is derived from the embryonic mesenchymal portion of the tooth, the dental papilla. Through a series of interactions with the cells of the epithelial layers of the developing tooth, the cells of the dental papilla differentiate into different cell types, one of which gives rise to the dentin that eventually surrounds the papilla, which is then termed the dental pulp (Ruch 1985).

The dental pulp consists of two major components: a cellular component and an extracellular matrix. Within the cellular component are many different types of cells.

The most distinctive of these cells, the odontoblasts, can be found along the periphery of the pulp, adjacent to the predentin layer. These cells consist of two parts, the cell body found within the pulp, and the odontoblastic process, found within the dentinal tubules. The cell body contains a basally-located nucleus (towards the pulp), a well-developed endoplasmic reticulum and Golgi complex, many ribosomes (associated with the reticulum), many mitochondria, a large number of secretory and coated vesicles and a highly developed microtubular system, which extends the full length of the odontoblastic process as well. The odontoblasts also have many complex junctions, especially where the process and the cell body meet. A system of zonula occludens and zonula adherens make up the terminal web, connecting all the adjacent odontoblasts. These organelles are indicative of the cells' function, which is production and subsequent calcification of the dentinal matrix. More details regarding the morphology and ultrastructure of the odontoblasts can be obtained from Ten Cate (1985) and Garant and Cho (1985).

The most numerous cell found in the dental pulp is the fibroblast. These cells can be found throughout the pulp, concentrated in the cell-rich subodontoblastic zone and in the apical portion of the tooth. Morphologically, these cells are similar to fibroblasts found in other connective tissues of the body. However, in the continuously growing

rodent incisor, various stages of fibroblastic differentiation can be seen. Young cells, found in the growing or apical end of the tooth are polygonal in shape, have little endoplasmic reticulum, few ribosomes (mostly in free form), a poorly-developed Golgi complex, few small mitochondria and few vesicles, all characteristic of immature, non-functioning cells (Han, Avery and Hale 1965). As these cells age, changes take place within them that reflect their changing function (Eifinger 1970; Baume 1980). The cells become stellate in shape with many cytoplasmic extensions, develop an extensive endoplasmic reticulum which has most of the abundant ribosomes associated with it, a well-developed Golgi apparatus with associated secretory vesicles and other organelles (microtubules, cilia) which are indicative of the cellular secretory function. This involves the initial secretion of the ground substance of the pulpal matrix, followed by the secretion of collagen fibrils (Eifinger 1970; Baume 1980). At the tip of the pulp, in what is termed the incisal end, the fibroblasts may be older and non-functioning. The cellular organelles have regressed, and the cells resemble the immature fibroblasts (Han, Avery and Hale 1965; Harris and Griffin 1967). The Golgi and endoplasmic reticulum have diminished and few ribosomes or vesicles are present. The cells may accumulate intracellular fibres and possibly dense bodies, but they (the

cells) have no proven function in the pulp (Harris and Griffin 1967; Eifinger 1970; Baume 1980).

A third cell type, the undifferentiated mesenchymal cell, can also be found throughout the pulp. These cells act as reserve cells and have the ability to differentiate into fibroblasts or odontoblasts to replace those cells that are damaged or have died. These cells are distinguished by their lack of granular endoplasmic reticulum or free ribosomes, abundant cytoplasmic processes and a large, centrally-placed nucleus. They may appear polyhedral or spindle shaped, due to the plane of section. The mesenchymal cells are concentrated in the subodontoblastic zone and around the pulpal blood vessels (Harris and Griffin 1969; Baume 1980; Ten Cate 1985).

The remaining cells found in the pulp are those of the vascular system (endothelial cells and pericytes), the neural elements (nerve cells and Schwann cells) and cells of the defense system. The last group includes lymphocytes, neutrophils, macrophages and mast cells, which may all be found in the pulps of healthy individuals. The main function of these cells is to digest cellular debris and protect the pulpal cells against infections, eg., carious bacteria. Also, when needed, other defense cells such as plasma cells and activated lymphocytes may be found in the pulp (Ten Cate 1985; Avery 1986).

The cell-rich subodontoblastic zone merits a special word on its cell types. This zone, present in both rodent and human pulps, is made up of a high density of cells just proximal to the odontoblastic cell bodies (Figure 1). The cells found within this zone are mainly fibroblasts and undifferentiated mesenchymal cells along with the cells of the neural and vascular elements (Harris and Griffin 1969). However, the fibroblasts within this zone are unique in that they have features that are similar to those of odontoblasts. As reported by Eifinger in 1970, and reviewed by Baume in 1980, these cells have extensive rough endoplasmic reticulum, abundant Golgi complexes and many secretory vesicles. They differ from the odontoblasts in their centrally-placed nucleus and scattered organelles, but they also differ from other pulp fibroblasts in their location, bipolar fusiform shape, and their specific function. These cells are thought to produce high amounts of ground substance and collagen, and also may be involved in the production of von Korff fibres (Baume 1980). The production of these fibres was once thought to contribute to dentin formation (Weidenreich 1925; Lester and Boyd 1968; Moss 1974) but this has recently been disputed (Ten Cate et al 1970; Ten Cate 1978). Also, the cells of this zone have the highest APase and Mg^{2+}-,Ca^{2+}-ATPase activity (as mentioned earlier), and the highest dehydrogenase enzyme activity (Goggins and Fullmer 1966, 1967) of all pulpal cells. This may be an indication

of the high synthetic activity or the presumed transport function of these cells. As well, it has been postulated that the cells of the subodontoblastic zone may help support the odontoblasts metabolically (Gotjamanos 1969b). This zone is also important in that it is from this pool that replacement cells for the odontoblasts arise. Both the fibroblasts and undifferentiated mesenchymal cells are thought to differentiate into odontoblasts when necessary (Trowbridge 1984; Yamamura 1985).

The second major component of the dental pulp is the extracellular matrix. It is produced mainly by the odontoblasts and the fibroblasts, the cells adapted for such production and secretion. This matrix consists of ground substance and collagen fibres. The ground substance is made up of many substances including connective tissue glycosaminoglycans, proteoglycans, glycoproteins, and water. Lipids also have recently been identified in the matrix (Linde 1985b). With regards to collagen, two types are found in the pulp. Type III is the predominant form in the developing pulp, but the amount of type I collagen increases as the pulp increases in age (Thesleff et al, 1979). Elastin also is found in the pulp, but only in the walls of the pulpal blood vessels. The components of the matrix can change over time. The matrix of a young pulp is different from that of an older pulp (Linde 1985b). For a

comprehensive review of the components of the pulpal matrix, see Baume (1980) and Linde (1985b).

The pulpal response to injury or exposure has been the subject of much investigation. This unique process of repair has been characterized by many investigators, but the general sequence of events has been agreed upon. Nyborg (1955) and Berman and Massler (1958) were the first to study the dental pulp healing processes in detail. Upon injury to the pulp, via exposure (due to pulpotomy or cavity preparation) or infection, a sequence of events takes place that is similar to other connective tissue healing processes. The trauma to the pulp must be sufficient to induce the reparative process. A low level stimulus will not induce healing of the defect (Granath and Hagman 1971). The destruction of odontoblasts, either by the cavity preparation or by the pulpal capping agent leaves a layer of necrotic debris on the surface of the exposed pulp. This is followed by an influx of inflammatory cells to help clear the debris and protect against infection. This is similar to the inflammatory reaction that normally accompanies wound healing. The migration and proliferation of pulpal cells is the next stage. These cells, identified as pulpal fibroblasts, undifferentiated mesenchymal cells and endothelial cells (Fitzgerald 1979; Yamamura 1985) migrate from the subodontoblastic zone (if undamaged) and the pulp proper. Yamamura (1985) believes that the fibroblasts and endothelial cells

undergo dedifferentiation to mesenchymal cells during this migration. These cells then proliferate and differentiate under an unknown influence into cells capable of matrix production. Fitzgerald (1979) believes that fibroblasts are the main replacement cells for the odontoblasts. These newly differentiated, odontoblast-like cells are then responsible for the formation of a fibrous, irregular dentinal matrix. This matrix, characterized by a lack of tubules, a decreased density, and many cellular inclusions has been termed osteodentin (Takuma et al 1968; Yamamura 1985). This "scar tissue", as Schröder (1985) termed it, protects the pulp from further irritation and destruction. This is followed by the formation of regular tubular dentin by newly differentiated odontoblasts. This entire process takes about 28 days to complete (Berman and Massler 1955; Yamamura 1985), although longer times (presumably for cellular renewal) have been reported (Schröder 1985). The stimulus for the cellular reaction (differentiation of cells into odontoblasts) is uncertain but a number of inducers have been proposed (Baume 1980; Yamamura 1985).

The abnormal production of osteodentin in the dental pulp, i.e., without previous tissue injury, has been seen with the administration of various chemotherapeutic agents. Vinblastine (Mikkelsen 1978), vincristine (Stene 1979; Stene and Koppang 1980), cyclophosphamide (Koppang 1978, 1981) and colchicine (Nogueira et al 1981) have all been shown to

cause osteodentin formation in rat incisors. Karim, in a series of papers (1984, 1985, 1986), has reported that the antibiotic Adriamycin (doxorubicin hydrochloride) also causes osteodentin formation in the incisors of rats. This phenomenon has also been confirmed by Dahl in a separate series of papers (1984, 1985). This drug, used clinically as an antineoplastic agent (Wang et al 1971; Bonadonna et al 1975), both alone and in conjunction with other chemotheraputics (Sutow 1975), has been demonstrated as having significant effects upon the skeletal system, the cardiovascular system (Young 1975), the bone marrow (Philips et al 1975), the urinary system (Olson et al 1974), and the peripheral nervous system (Cho 1977). It has also been found to be teratogenic when administered to pregnant rats on days 6 to 15 of gestation (Thompson et al 1978).

Experiments have shown that on the day following administration of the drug, a lesion of necrotic cells appears (Karim 1985a) that extends for about 2.2 mm into the apical end of the incisor pulp (Karim and Pylypas 1985). The pulp cells affected were the preodontoblasts and their precursors, cells that may be found in the subodontoblastic zone of the apical end of the pulp (Karim 1985a; Dahl and Koppang 1985). Cells that had matured to the point of initial dentinal secretion (mantle dentin) were insensitive to the drug's effects (Karim 1985a; Dahl and Koppang 1985). Three days after adriamycin injection, pulp mesenchymal

cells were seen to aggregate near the site of subsequent osteodentin formation, just incisal to the area of the lesion. These cells also were seen to have differentiated into odontoblast-like cells, capable of secreting the osteodentin matrix, which appeared between days 3 and 7, post-injection (Karim 1985b). Ultrastructural observations of the matrix showed that it was a collagenous matrix that was similar in some respects to that of predentin and dentin (Karim 1985b). Using radioautography, Karim and Pylypas (1986) demonstrated that a component of this collagenous matrix was indeed synthesized by these abnormally differentiated cells. The ^{3}H-labelled proline was seen over the cells at 5 minutes after injection and over the matrix at as little as 1 hour after injection. The osteodentin formation, which begins at the periphery and continues towards the center of the pulp, has been seen to occlude the entire pulp chamber in some regions of the tooth (Karim and Eddy 1984). Other effects of adriamycin upon the rat incisor include reduced dentin formation by the odontoblasts (Dahl 1984; Dahl and Koppang 1985), abnormal dentin formation, and a failure of the closure of the apical foramen (Karim, unpublished results).

The present study was undertaken to examine the APase and Mg^{2+}-,Ca^{2+}-ATPase activity of the cells of the rat incisor pulp prior to and following the administration of Adriamycin, and to determine what relationship, if any, exists between the presence of the enzymes and the differentiation of pulp cells into odontoblast-like cells.

MATERIALS AND METHODS

Animals

All animals used in this study were obtained from the Dental Animal Care facility, where they were also housed and cared for during the study period. A total of 34 young (10 days old) male Sprague-Dawley rats (30 ± 3 g) were used. They were divided into 5 groups. Group A consisted of 4 rats that were used to study normal pulp morphology. Group B consisted of 8 rats used to study the normal alkaline phosphatase activity in untreated pulp tissue. Groups C and D consisted of 16 and 3 rats respectively, and they were used to study the alkaline phosphatase activity of Adriamycin-treated pulps. Group E, which consisted of 3 animals, was also used to study the alkaline phosphatase activity of Adriamycin-treated pulps, but employed a different method than that of groups C and D.

Injection of Animals

Groups A and B: The animals were sham injected with 0.1 cc of physiological saline so as to study the normal pulp morphology and normal enzyme activity of the pulp, and to determine whether any subsequent changes could be due to the trauma of injection.

Group C: The rats were subcutaneously injected with Adriamycin (Adria Laboratories of Canada, Ltd.) at a dose of

5.0 mg/kg. This has been shown to be a dose consistent with osteodentin production in the pulp (Karim 1985; Karim and Pylypas 1986). Subgroups of 6 rats each were sacrificed 3 and 5 days after administration of the drug. In the 7 days after administration subgroup 2 animals died prematurely due to undetermined causes; thus only 4 animals in this group were sacrificed.

Groups D and E: The animals were treated in the same manner as those in group C except all of them were sacrificed 7 days after Adriamycin administration, a period consistent with advanced osteodentin production (Karim and Pylypas 1986).

Perfusion of Animals

Perfusions of all the animals were done using the formaldehyde solution (Appendix) described by Granström et al (1978) as being optimal for the preservation of alkaline phosphatase activity. The rats were anesthetized by ether inhalation and perfused through the left ventricle for 15-20 minutes using the formaldehyde solution. The jaws were dissected out of the rat and immersed in the perfusion solution for an additional 1-2 hours. The total number of teeth in groups B and C were divided and one half put into an EDTA solution for decalcification. All of the teeth in group E also were demineralized. The pulps of the remaining incisors of each group were dissected out and washed in

0.15 M cacodylate buffer (pH 7.2) overnight. These pulps were then cut into 1 mm-thick cross-sectional segments and separated according to location (apical, middle and incisal) within the incisor.

Decalcification

The incisors of groups B, C, and E to be decalcified were rinsed with cacodylate buffer and placed in a 4% EDTA solution (Appendix) at 4°C with continuous stirring for a period of one week. The solution was changed every 2 days during this period. The material was subsequently rinsed in cacodylate buffer and cut into 1 mm-thick cross-sectional segments. These pieces were separated according to location (apical, middle and incisal) within the incisor, and those of groups B and C were placed in a 0.1 M $MgCl_2$-Tris-Maleate solution (pH 7.4) at 4°C for two hours in order to reactivate the alkaline phosphatase enzyme (Orams and Snibson 1982). The pieces in group E, processed according to the method of Takano et al (1986) were not reactivated following decalcification. Prior to incubation, all of the decalcified material was washed in cacodylate buffer.

Incubation

Group A: The pulps were not incubated in any solution, in order to process and study them for normal pulp morphology.

Groups B and C: The material (pulps and decalcified segments) was separated into 4 groups and treated in the following manner:

1) incubated in a medium described by Granström et al (1978) (Appendix).

2) heated in buffer at 56 °C for 15 minutes to inactivate the enzyme and incubated in a medium similar to 1) but not containing the substrate, ATP.

3) incubated in a medium similar to 1) but also containing 0.1 mM levamisole (Aldrich Chemical Co.), an inhibitor of non-specific alkaline phosphatase (APase).

4) incubated in a medium similar to 1) but also containing 0.1 mM ouabain (Sigma Chemical Co.), an inhibitor of sodium-, potassium-adenosine triphosphatase (Na^+-,K^+-ATPase).

The pulps and decalcified sections were incubated separately but for the same time periods of 10, 20 and 30 minutes at room temperature (20 °C).

Group D: The material (pulp sections) was also treated in the same manner as groups B and C, but for time periods of 30 and 60 minutes at room temperature (20 °C).

Group E: The tissues were treated similarly to those in groups B, C, and D, but using different incubation solutions as described by Takano et al (1986) (Appendix). They were divided into 4 groups and treated as follows:

1) incubated in the above mentioned medium.

2) heated in cacodylate buffer at 56°C for 15 minutes to inactivate the alkaline phosphatase and incubated in a medium similar to 1) but not containing ATP.

3) incubated in a medium similar to 1) but also containing 0.1 mM levamisole.

4) incubated in a medium similar to 1) but also containing 0.25 mM ouabain.

This material was incubated for periods of 30 and 50 minutes at 37°C.

After incubation, all tissue was washed with cacodylate buffer and processed.

For a summary of this section, see Table 1.

Processing

All tissue samples (groups A-E) were processed in a similar manner.

The washed samples were post-fixed in 2% aqueous osmium tetroxide solution (Appendix) at room temperature (20°C) for 1 hour.

The post-fixed material was dehydrated in graded concentrations of aqueous acetone as follows:

Acetone concentration	Time
30%	5 min. (1 change)
50%	5 min. (1 change)
70%	5 min. (1 change)
80%	5 min. (1 change)
90%	5 min. (1 change)
100%	5 min. each (3 changes)

After the final change in pure acetone, the samples were placed in a 3:1 acetone-Epon mixture (Appendix) to initiate the infiltration process. The following schedule was employed:

Acetone:Epon	Time
3:1	12 hours
1:1	24 hours
1:3	12 hours
Epon only	12 hours
Fresh Epon	4 hours

The tissue was embedded in a freshly made Epon mixture. Beem capsules were partially filled with Epon, and the sections (one per capsule) were oriented such that the cut cross-sectioned surface was facing the bottom of the capsule. The capsules were filled with Epon and then placed in a 60 ^{o}C oven for 2-3 days for polymerization.

Sectioning

Blocks were taken at random from each of the groups. The blocks were trimmed, and a series of ultrathin sections (pale gold interference color) from each block were cut on a Reichert OM U3 ultramicrotome using a glass knife or a diamond knife (for undecalcified sections). These sections were placed on uncoated 300 mesh copper grids for observation.

Staining

Some of the grids prepared were contrast stained using uranyl acetate (Appendix) and lead citrate (Appendix) solutions.

Uranyl Acetate

The solution was drawn from midway in the bottle (to avoid precipitate) and filtered directly on to a waxed staining plate. The grids were placed section-side down upon the surface of the drops for 10 minutes. After staining, the grids were washed in a stream of distilled water and then rinsed using straight up and down movements in 3 changes of distilled water. They were dried on filter paper.

Lead Citrate

The solution was filtered and dropped directly on the staining plate, which was in an atmosphere of sodium hydroxide to avoid precipitation. The previously UA-stained grids were placed section-side down upon the drops for 15 minutes. They were washed, rinsed and dried as described above.

Electron Microscopy

Observations (on both stained and unstained grids) were made on a Hitachi HU-12 electron microscope operated at 75 kv.

Table 1

Summary of the media and times from the Incubation portion of Materials and Methods.

	GROUP A Untreated	GROUP B Untreated	GROUP C 3 Days after Adriamycin	GROUP C 5 Days after Adriamycin	GROUP C 7 Days after Adriamycin	GROUP D 7 Days after Adriamycin	GROUP E 7 Days after Adriamycin
Granstrom, Linde, and Nygren (1978) <u>MEDIUM</u> Tris-maleate buffer, pH 9.3 Na+ K+ Ca++ ATP Cadmium acetate <u>Reactivation solution</u> Tris-maleate buffer, pH 7.4 $MgCl_2$	Not Incubated	1) Medium 2) Heat inactivated 56°C, 15 min. medium without ATP 3) Medium with levamisole 4) Medium with ouabain Times of 10, 20, 30 min. <u>EDTA-treated</u> reactivated 2 hours 1),2),3),4) as above Times of 10, 20, 30 min.	1) Medium 2) Heat inactivated 56°C, 15 min. medium without ATP 3) Medium with levamisole 4) Medium with ouabain Times of 10, 20, 30 min. <u>EDTA-treated</u> reactivated 2 hours 1),2),3),4) as above Times of 10, 20, 30 min.	1) Medium 2) Heat inactivated 56°C, 15 min. medium without ATP 3) Medium with levamisole 4) Medium with ouabain Times of 10, 20, 30 min. <u>EDTA-treated</u> reactivated 2 hours 1),2),3),4) as above Times of 10, 20, 30 min.	1) Medium 2) Heat inactivated 56°, 15 min. medium without ATP 3) Medium with levamisole 4) Medium with ouabain Times of 10, 20, 30 min. <u>EDTA-treated</u> reactivated 2 hours 1),2),3),4) as above Times of 10, 20, 30 min.	1) Medium 2) Heat inactivated 56°C, 15 min. medium without ATP 3) Medium with levamisole 4) Medium with ouabain Times of 30, 60 min. No EDTA-treatment	Not Incubated
Takano, Ozawa, and Crenshaw (1986) <u>MEDIUM</u> Tris-maleate buffer, pH 8.2 Glucose Lead citrate Ca++ ATP	Not Incubated	Not Incubated	Not Incubated	Not Incubated	Not Incubated	Not Incubated	All EDTA-treated No reactivation 1) Medium 2) Heat inactivated 56°C, 15 min. medium without ATP 3) Medium with levamisole 4) medium with ouabain Times of 30, 50 min.

RESULTS

A diagram demonstrating the location of some of the cells and the relevant features of the pulp of the rat incisor discussed below can be seen in Figure 1.

Normal Pulp Morphology (Group A)

Pulp cells were more numerous in the apical end of the pulp and became less numerous towards the incisal end. A corresponding increase in the number and density of fibrous material in the matrix towards the incisal end was also seen. Undifferentiated mesenchymal cells were seen throughout the pulp but were more common in the subodontoblastic zone. These polyhedral-shaped cells were characterized by the absence of granular endoplasmic reticulum and lack of free ribosomes in the cytoplasm. Few mitochondria were present and some vesicles were seen, but no other organelles were found. Blood vessels (made up of endothelial cells) were also found throughout the pulp, but these will be described below. Odontoblasts were seen in all sections but are not described in detail, since this has been well documented by other investigators (Garant and Cho 1985; Holland 1985; Ten Cate 1985).

Apical

The pulp cells in the apical end of the incisors were very numerous. They were rounded to polygonal in shape and possessed few organelles. Little granular endoplasmic reticulum (ER) but many loose ribosomes in the form of rosettes were seen. Good profiles of Golgi apparatus were present, and some vesicles were associated with them. Membraned multivesicular bodies (MVBs) were found within some cells but were not common. Mitochondria were present but were few in number. Those that were observed were small, with poorly developed cristae. Cilia and centrioles were in some cells, but also were not common. Glycogen granules were present in many of these cells. The cytoplasm of the cells was otherwise empty-looking. The nucleus was large and generally contained only one nucleolus. The pulpal matrix was also unremarkable, containing only strands of a fine amorphous material and few fibrils scattered between the cells (Figure 2).

Middle

The cells in the middle of the pulp were more mature and resembled functioning secretory fibroblasts (Figure 3, 4). They were polyploid in shape and possessed cellular extensions. The cells also contained more organelles. Extensive granular ER was seen, and many of these profiles were dilated. The number of vesicles associated with the

Golgi apparatus was increased, and MVBs were more common. Coated vesicles were also present in many cells. Mitochondria were increased in both number and size, most having well-developed cristae. They also contained matrix granules in association with these cristae. A microfilament network had developed within some of the cells, and centrioles and cilia were observed, occasionally in the same cell. As well, dense bodies were found in the cells, especially those located closer to the incisal end. The nucleus was smaller in relation to the whole cell size and enclosed in a well-developed, double-membraned nuclear envelope with ribosomes along the outer membrane. Cell-to-cell junctions, especially desmosomes, were seen between adjacent cells. An interesting observation was that two different types of pulpal cells were present. There were some that had numerous profiles of granular ER and a well-developed secretory apparatus (Figure 3), and there were others that contained reduced granular ER but many profiles of Golgi apparatus and vesicular bodies (Figure 4). The former cells were associated with the fibrous materials of the matrix. Some of the fibrils were seen attached and passing into these cells (Figure 3). The latter type of cells were not associated with these fibrils (Figure 4). However, the amount of amorphous material found in the pulpal matrix had also increased. The matrix contained more cut profiles of cells and cellular extensions.

Incisal

The pulpal cells in the incisal end of the incisor were much the same as those in the middle section. They had essentially the same features (granular ER, Golgi apparatus, vesicles, mitochondria, etc.) and characteristics as those mentioned above (Figures 3, 4). The two different cell "subtypes" were also seen: those with much granular ER, and those with decreased ER but increased Golgi and vesicular profiles. All of the cells showed some microfilamentous structure within the cytoplasm. Coated vesicles were encountered in more cells, especially those containing many vesicular bodies. Desmosomes were again present between adjacent cells. Rarely, cells having few or poorly developed organelles and an increased number of microfilaments and dense bodies were seen. The granular ER was reduced, and few rosettes were present. The Golgi apparatus was reduced in size, and there were few vesicular structures present. The number of mitochondria was reduced, and those seen had small cristae. The cell size was smaller than other pulpal cells, and the cell shape was more oval, with fewer extensions. The matrix of the incisal pulp was quite fibrillar, and contained vast amounts of fine amorphous material, usually seen in clumps. The cells having numerous profiles of granular ER were again associated with these fibrils, either through their close proximity or actual contact with their membranes. Some fibrils were seen to

pass into cells and continue within the cytoplasm. A few cells had the fibrils sequestered in their cytoplasm (Figure 5).

Throughout all areas of the pulp, cells located in the subodontoblastic (SO) zone were different from other cells of the pulp. Undifferentiated mesenchymal cells found in this zone were the same as those found in any area of the pulp, but the pulpal fibroblasts were further differentiated than pulpal fibroblasts found elsewhere in the pulp. They were somewhat polarized, having their nucleus in one end of the cell. They also appeared to contain greater quantities of dilated granular ER, coated vesicles, and mature mitochondria. The Golgi and vesicular profiles were similar to other pulp cells, and there were MVBs present in the cytoplasm. Other organelles were similar to those seen in the cells of the middle third of the pulp. Numerous cellular extensions were seen, and these were often in contact with the fibrils or amorphous material of the surrounding matrix, which also contained some cross-banded collagen fibres were present. As well, the concentration of cells was always higher in the SO zone than in the rest of the pulp.

Blood vessels seen in the pulp and the SO zone were like those seen in other connective tissues (Figures 19-22). All of the vessels seen in these pulps consisted of cross-sections of two to three endothelial cells connected to each other via cellular junctions. Organelles of the cells

(granular ER, Golgi apparatus, mitochondria, etc.) were present, and some pinocytotic or endocytotic vesicles were seen near the luminal surface. Few structures were seen in the luminal space, and undifferentiated mesenchymal cells or pericytes were sometimes associated with the periphery of the blood vessels.

Normal Alkaline Phosphatase Activity of Pulp (Group B)

The sections in group B were incubated for times of 10, 20, and 30 minutes. The reaction appeared as a dark precipitate. As the time of incubation increased, there was a concomitant increase in reaction intensity. A wide range of reaction intensities was observed as different sections from the same block were examined, i.e., those sections from the surface of the block had a more distinct and intense reaction than those from deeper areas in the same block. The demineralized (EDTA-treated) material exhibited a somewhat less intense reaction, even with the longest incubation time, in comparison to the undemineralized material. In some demineralized sections, no reaction was seen. Only the mature pulpal fibroblasts and the endothelial cells of the blood vessels in any of the three regions of the pulp exhibited enzyme activity. The immature fibroblasts and the undifferentiated mesenchymal cells had no reaction associated with them, regardless of the region of the pulp in which they were located. Therefore, the enzyme activity was most

evident in the functioning pulpal fibroblasts, located primarily in the middle, apical, and SO zones of the pulp. The fibroblasts of the SO zone exhibited the most intense reactions, and form the bulk of the observations noted below. The components of the pulpal matrix exhibited no reaction in any of the sections, regardless of the pulpal region or the incubation medium.

The sections from the blocks incubated in **medium 1** showed a variety of reactions. As was mentioned above, the longer the period of incubation, the greater the intensity of reaction. The components of the cell secretory pathway exhibited the greatest enzyme activity. The granular ER was most evident in the cells, especially in the non-contrast stained sections (Figures 6, 7, 11). The next most evident site of reactivity was seen in association with the vesicular structures. Most of the reaction was seen along their membranes and rarely within the vesicles (Figures 6, 7-9). Coated vesicles and MVBs also exhibited some reaction, again mainly along the membranes (Figures 6, 11). The Golgi apparatus showed very little reaction, even when seen at high magnification (Figures 6, 10b). Contrast-stained sections camouflaged any enzyme activity sites, and no conclusive observations could be made (Figure 10a). On the other hand, unstained sections demonstrated the lack of enzyme activity sites quite readily (Figures 6, 7, 11). Mitochondria exhibited some reaction along the outer

membranes and the cristae. This was most evident at longer incubation times (Figures 6, 11, 12). The nucleus and cytoplasm rarely showed sporadic, scattered precipitate, but it was not concentrated in any one area and is probably due to a slight background reaction. Finally, cell membranes (Figures 9, 11) and membraned structures resembling empty cellular extensions (Figure 12) demonstrated some reaction.

In the sections that were heat-inactivated and incubated in **medium 2**, very little or no enzyme activity was observed. Only in the tissues incubated for 30 minutes was any reaction seen, and this was a very weak one along the granular ER. No other organelles of any cells exhibited a reaction, and none was present in the pulpal matrix (Figure 13).

The material that was incubated in the medium containing the levamisole (**medium** 3) exhibited a more specific enzyme activity than that of medium 1. Very little reaction was associated with the pulpal cells or their organelles. The reaction along the granular ER was reduced and again, no reaction was seen on the Golgi saccules (Figures 15, 17). The reaction associated with the mitochondria was also reduced. Some of the vesicles, especially the uncoated ones, exhibited reactivity (Figures 14, 16, 17). Multivesicular bodies and coated vesicles only had occasional reaction deposits (Figures 14, 16). Even at 30 minutes incubation time, the reaction in association with vesicular components was slight.

Incubation of sections in the presence of ouabain (**medium 4**) yielded results similar to those seen in sections incubated in medium 1. Reactions were seen along the granular ER, the vesicles, and the MVBs, but no reaction was seen in association with the Golgi apparatus or the mitochondria of the pulpal cells. The granular ER, the vesicles, and MVBs also demonstrated reaction precipitate within the SO fibroblasts, but again, no reaction was seen in association with the Golgi apparatus or the mitochondria of these cells (Figure 18). In fact, no mitochondrial reaction was seen in any cells of the pulp.

The endothelial cells of the blood vessels found in the pulp also exhibited alkaline phosphatase activity. The activity was quite evident in sections incubated in **medium 1.** The vesicles located along the luminal and abluminal membranes showed the most intense reaction (Figures 19-22). The cellular membranes also showed some reactivity, especially along the luminal surfaces (Figures 19, 21). Other organelles exhibiting reaction were the granular ER and the few mitochondria found in the cytoplasm (Figure 21). No activity was associated with the endothelial cells seen in sections that were heat-treated and incubated in **medium 2.** Few blood vessels were observed in the sections incubated in the presence of levamisole (**medium 3**), but those seen exhibited slight reactions only along the granular ER and mitochondria of the endothelial cells. The sections

incubated in **medium 4** again contained few blood vessels, but the reactions associated with the endothelial cell organelles (cell membranes, vesicular structures, and granular ER) were as intense as those observed in medium 1-incubated sections. The reactivity associated with the mitochondria, however was completely absent in the presence of ouabain.

Effect of Adriamycin on Pulp (Groups C, D, and E)

The administration of Adriamycin caused abnormalities similar to those seen by Karim (1984, 1985a) and Dahl (1984). Three days after treatment, changes were seen only in the apical end of the pulp. Necrotic cells were found along the labial side of this region, but few defense cells were present. Those observed were generally peripheral blood granulocytes. Just incisal to this area, a change in the cells of the peripheral pulp, including those of the SO zone, was noticed. The cells were enlarged, and the cell density was higher than in the control pulps. The cells were similar to SO fibroblasts, but changes in the cellular shape and organelles were seen. The free ribosomes had increased in number, as had the amount of granular ER. The number of vesicles in the cells had increased, and the number of mitochondria present was also higher. The cells took on an elongated shape with the nucleus displaced to one end, and cytoplasmic extensions had developed (Figure 23). These cells resembled early odontoblasts and were termed

odontoblast-like cells, similar to those described by Karim (1984, 1985a). In addition to these cells, a few dying or degenerating cells having large vacuoles in the cytoplasm were seen. The matrix surrounding these odontoblast-like cells was unlike the normal matrix seen in this zone. Large amounts of fine amorphous material, microfilaments, and fibrils were observed in association with these cells (Figures 26, 28). Also, collagen fibres exhibiting cross-banding were seen (Figures 25, 26, 28). Much of the fibrillar material was membrane-bound within and in close approximation to cut cellular profiles or extensions (Figures 26, 28). The arrangement of this matrix was quite irregular, with collagen fibres and fibrils crossing each other without any apparent pattern or alignment. Karim (1985a) has previously called this area the site of initiation of osteodentin formation. No crystalline material was observed and no evidence of mineralization was found in any of the sections containing this fibrillar-cellular arrangement. As well, matrix vesicles commonly associated with initial hard tissue formation were not seen in any of these sections.

Five days following the drug treatment, the number of necrotic cells was less and no defense cells were found in the pulp. The osteodentin deposit was again located in the apical portion (labial side) of the pulp, and was increased in size. The matrix now was more fibrous and less fibrillar, with the collagen fibres seen in cross, oblique, and

longitudinal section (Figure 32). It consisted of 2 areas; a dense central portion containing the irregularly arranged collagen fibres, and a less dense outer portion containing irregularly arranged fibrils with some fibrous material present. These two layers resembled the dentinal and predentinal matrices, respectively, but the osteodentin was atubular and not as dense as dentin and predentin. Both of these observations are similar to those made by Karim (1985a). In the undemineralized sections, areas of mineralization of the dense central matrix were seen. These mineralized portions also appeared to be less dense than those of normal circumpulpal dentin. As well, some sections of the crystals that make up this matrix were observed. These crystals resembled long, thin rectangles that did not twist about their long axis. They too were irregular in arrangement, with some visible overlap occurring between adjacent rectangles (Figure 31). Many odontoblast-like cells were associated with osteodentin, either within (surrounded by the fibrous matrix) or on the periphery of the deposits (Figures 33, 34). These cells were similar to those seen in the 3 day sections, except they were more developed and looked more like mature secretory cells. They had more profiles of granular ER and vesicular structures, including evidence of secretion granules in the cytoplasm (Figure 35), but they were not as polarized or compartmentalized as odontoblasts. However, they did have a number of

cytoplasmic extensions and again, were found surrounded by the matrix, making them similar to osteoblasts found in bone. Many of these odontoblast-like cells were seen in close association with the collagenous fibres of the matrix. In this association, the fibres were either in close contact with the cell membrane, or were seen inside the cytoplasm.

The pulps treated with Adriamycin for 7 days were from groups C, D, and E. Very few necrotic cells were found in the pulp, and no defense cells were seen, but the area just apical to the osteodentin was empty-looking, with few cells or cellular structures present. The osteodentin deposit was larger, and more mineralized tissue was present in comparison to that of the 5 day sections. It was also located along the labial edge of the apical end of the pulp, but was slightly more incisal than that seen in the 3 day sections. The characteristics of this deposit were much the same as previously presented. The matrix itself was a dense and highly irregular fibrous meshwork, and like that seen in the 5 day sections, resembled the predentinal and dentinal matrices of normal teeth (Figure 38), but the osteodentin was again atubular and less dense. Cellular inclusions were again observed throughout the osteodentin deposit, with some of these undergoing autolysis. The odontoblast-like cells had characteristics similar to those seen in the 5 day pulps. The cells were slightly elongated with cellular extensions, some found within the matrix (Figure 43).

Extensive granular ER was seen, most of it dilated (Figure 38). A number of profiles of the Golgi apparatus were present, as were a number of vesicles. Secretion granules, similar to those in odontoblasts, also were seen in these cells (Figures 39, 42). Other organelles (mitochondria, MVBs, etc.) were found within these cells as well.

Endothelial cells in the treated pulps showed no changes due to the drug treatment. However, blood vessels were in close proximity to the odontoblast-like cells and the osteodentin deposits in both the 5 day and 7 day material. Some were even seen within the matrix in these sections.

Effect of Adriamycin on Alkaline Phosphatase Activity of Pulpal Cells (Groups C, D, and E)

Following the Adriamycin treatment, the pulpal fibroblasts (including those of the SO zone) and the undifferentiated mesenchymal cells in sections from groups C, D, and E exhibited no change in enzyme activity from those seen in group B. Most of the enzyme activity was observed in association with the cells of the osteodentin deposit, which was always in the apical end of the pulp, but no activity other than that noted in the pulp cells was seen in the middle or the incisal pulpal regions. A great variety of reactions was seen between sections showing different areas of the pulp. Cells in one area would show a heavy reaction,

while the same type of cell in another area, from a section cut deeper in the same block would show less reactivity. As well, some variability occurred in different parts of the same cell, especially when cut tangentially. The cells in the demineralized (EDTA-treated) sections were found to have diminished reactions, and, in some cases, none at all in comparison to the undemineralized sections. Also, as stated above, the increase in the times of incubation increased the intensity of the reaction. The observations of the 3 day and 5 day sections are from group C pulps, while those of the 7 day sections are from groups C, D, and E, with some explanation as to the differences between the groups.

Three Days After Administration

The odontoblast-like cells showed greater alkaline phosphatase activity than the unaffected pulpal cells or those of the group B sections. This was quite evident in sections incubated in **medium 1**. The organelles involved in protein synthesis and secretion exhibited the heaviest reactions (Figure 23). The Golgi apparatus, however, had no reaction associated with it. Some vesicles that resembled secretion granules of odontoblasts demonstrated reactions, especially when observed in cellular extensions in the developing osteodentin matrix (Figures 24a, 24b, 25), as did other structures, most likely MVBs (Figures 25, 27a). Mitochondria of these cells contained little evidence of any

reactivity (Figure 26), even when incubation times were 30 minutes. Background reaction was not present in any section in this group. Membraned structures containing fibrils and amorphous material showed some reaction along their external membranes, and this was evident even with contrast-staining of the section (Figures 26, 28). No reaction was associated with the osteodentin matrix other than that of the membraned structures. Dying cells, having large vacuoles and few organelles, had heavy reactions associated with their cellular and vesicular membranes. These cells, few in number, were found around and within the developing osteodentin matrix.

Cells from sections that were heat-inactivated and incubated in **medium 2** exhibited no enzyme activity, as was seen in group B sections. The odontoblast-like cells showed slight reaction along the granular ER in those sections incubated for 30 minutes, but no other reaction was observed in the pulpal matrix or pulpal cells. The osteodentin matrix was also devoid of any reaction.

The sections incubated in **medium 3** contained cells showing a more specific enzyme activity. The granular ER reaction was diminished but not absent (Figure 29), and the vesicles and MVBs demonstrated reactions along their membranes. Some of the vesicles found in the cellular processes resembled secretion granules of odontoblasts, and were evident because of the reaction associated with them.

The mitochondria and Golgi apparatus demonstrated no reaction, and only a slight scattered reaction was seen along the cellular membranes and membraned structures found in the osteodentin matrix. No reaction was seen in direct association with the components of the matrices, pulpal or osteodentinal.

The results of the sections incubated in the presence of ouabain (**medium 4**) were similar to those seen in group B. The fibroblasts of the SO zone and the odontoblast-like cells of the osteodentin matrix exhibited activity similar to that seen in sections incubated in medium 1 (Figures 30a, 30b). The granular ER (Figure 30b), the vesicles, and the MVBs all demonstrated some reactivity. The Golgi apparatus showed no reaction, identical to that seen in the sections from medium 1. The mitochondrial reaction was absent, but this was also seen throughout the cells of the sections incubated in the other mediums (Figure 30c). Again the matrices, pulpal and osteodentinal, were free from reactions, except for the presence of cellular extensions within the developing osteodentin which exhibited some reaction precipitate along their outer membranes.

In summary, only the cells associated with the osteodentin matrix showed any change in alkaline phosphatase activity from that of the group B sections. Almost all activity was inhibited by the heat treatment. The granular ER reaction was diminished by the levamisole, as was that of

the vesicles and MVBs. The levamisole had little effect upon the reaction of the secretion granules. Golgi and mitochondrial reactions were absent in all sections, and only a slight membrane reaction was seen throughout the sections. Ouabain apparently had little effect upon the enzyme activity of any of the cells.

Five Days After Administration

Five days following administration of Adriamycin, the number of necrotic cells found in the pulp was much less, and no defense cells were seen. It was again found that the enzyme activity of all the cells was diminished or absent in the demineralized (EDTA-treated) sections.

The odontoblast-like cells of the sections incubated in **medium 1** again exhibited activity on organelles of the secretory pathway. The granular ER reaction was strong, and was even noticable in some EDTA-treated sections (Figure 33). The Golgi apparatus again exhibited very little or no reaction. The vesicles demonstrated a definite reaction, especially in the cells found within the osteodentin deposit and close to the mineralized portions. This reactivity was seen along the external membranes of these vesicles and MVBs found in the cells or cellular extensions. Secretion granules, more commonly seen in these cells than in the 3 day sections, demonstrated some reaction. Again, no reaction was observed on mitochondria, even at 30 minutes

incubation time. As well, no reaction was seen on any other organelles in the cells. Dying cells having reaction along their vacuoles and membranes were seen, both around the periphery of the osteodentin and occasionally within the deposit itself.

The material that was heat-treated prior to incubation in **medium 2** again showed very little enzyme activity. As previously stated in the 3 day sections, no organelles of any cell, with the exception of the granular ER of the SO fibroblasts and odontoblast-like cells, exhibited any reaction. As well, the observed precipitate along the granular ER of these cells was slight and sporadic (Figure 36).

The enzyme activity of the cells incubated in **medium 3** was intense enough to be observed on some EDTA-treated as well as undemineralized sections. The odontoblast-like cells exhibited the greatest activity along the granular ER and secretion granules (Figure 35). Although it was slightly less intense, the granular ER reaction was not greatly affected by the presence of levamisole (Figure 34). The secretion granules found in the cells demonstrated reactions unaffected by the levamisole in the incubation medium (Figure 35). Most of these were seen in the cells or their processes associated with the osteodentin matrix. The activity of the vesicles was somewhat affected, as only some of the vesicles and MVBs had reactions along their external

membranes. A few vesicles were seen in the matrix, near the mineralization front, and some of these were in close association with the processes of the odontoblast-like cells. Neither the Golgi apparatus nor the mitochondria presented any reaction (Figure 35). The membranes of the cells and the cellular processes showed some reaction. As stated earlier, the SO fibroblasts of the remainder of the pulp also exhibited some reaction precipitate, especially along the granular ER and vesicles, but not as intensely as the odontoblast-like cells.

The presence of ouabain in incubation **medium 4** seemed to have little effect upon the intensity of reaction on the organelles, although demineralization with EDTA reduced this intensity (Figure 37). The results are identical to those seen in sections incubated in medium 1 above. The granular ER, the vesicles, MVBs, and the secretion granules all had some reaction evident. The Golgi apparatus and the mitochondria in these cells demonstrated no reaction. Some vesicles and cellular extensions in the osteodentin matrix also exhibited enzyme activity uninhibited by ouabain.

In summary, the enzyme activity of the odontoblast-like cells in the 5 Day sections demonstrated the following reaction pattern. The heat-treatment of the tissue samples inhibited all enzyme activity, except for a bit on the granular ER of the cells. The granular ER reaction was otherwise only slightly diminished by levamisole. The

vesicular reaction (including that of the MVBs) was diminished by the levamisole, and only a few vesicles with reaction could be seen. The secretion granules increased in number and their enzyme activity was virtually unaffected by levamisole. The Golgi apparatus and the mitochondria of the cells again demonstrated no reactivity in any section, but the cellular membranes showed some reaction. Ouabain again seemed to have no effect upon the enzyme activity of any of the cells.

Seven Days After Administration

The results seen in these sections were very similar to those of the 5 day treatment set, except longer incubation times and a different set of incubation media were used. In group D, the material was incubated for 30 and 60 minutes. The extended incubation times increased the amount of background reaction, and this was quite evident in the 60 minute sections. However, this reaction was seen only over the cells, not on the matrix between the cells (Figure 39). Thus, only cellular structures exhibited any enzyme activity. The pulps from group E were prepared and incubated following a different method (Takano et al 1986, see Materials and Methods), but the incubation media used were analogous to those used in groups C and D. The same heat-inactivation and specific inhibitors were used so that these results could be compared to those of the above groups. Incubation

times for these sections were 30 and 50 minutes, and the same characteristics were seen: increased intensity of reaction, and increased background reaction. The background reaction seen in these sections, however, was on both the cells and the matrix. The results of the group C and D material are presented first, followed by those of the group E material.

Incubation in **medium 1** demonstrated definite enzyme activity on organelles of the odontoblast-like cells. The components of the secretory pathway (the granular ER, vesicles, and secretion granules) exhibited visible reaction precipitates (Figures 38, 39), but the Golgi apparatus again did not appear to have any reaction associated with it, regardless of the period of incubation (Figures 38, 39). In group D, the reaction on the granular ER of the sections incubated for 60 minutes was diminished and sometimes absent (Figure 39). As well, vesicles were distinctly outlined along their membranes in the cells and their extensions within the matrix (Figure 39). The secretory granules demonstrated intense reactivity, and they were quite distinct in the odontoblast-like cells incubated for 60 minutes (Figure 39). Multivesicular bodies and coated vesicles also demonstrated a slight reaction along their membranes. Mitochondria again exhibited no reaction on its membranes or cristae in cells from either group C or D (Figures 38, 39). There was some concentration of reaction along the cellular

membranes (Figure 39), which were sometimes seen in cross-section in the osteodentin matrix.

The results of the sections of groups C and D that were heat-treated and incubated in **medium** 2 were identical to those previously presented in the 3 day and 5 day sections. The only reactions seen were along the granular ER of the SO fibroblasts and odontoblast-like cells, and these were quite diminished and sometimes absent. No other cellular organelle showed any reaction.

In group C and D sections, the presence of levamisole in **medium** 3 had some effect upon the enzyme activity seen in the odontoblast-like cells. The granular ER reaction was slightly less than that seen in cells incubated in medium 1 for up to 30 minutes, but was absent in cells in the 60 minute incubation group. The reaction seen along the membranes of vesicles and MVBs was slight, but the reaction on the secretion granules was very intense, especially in the 60 minute incubation group. This reaction was most evident in cells near the mineralization front of the deposit. No reaction was seen along the saccules of the Golgi apparatus or the mitochondrial membranes and cristae of these odontoblast-like cells. Finally, the cellular membranes and the coated vesicles also demonstrated some reaction precipitate.

The sections incubated in **medium** 4 had results identical to those treated in incubation medium 1. The

odontoblast-like cells in groups C and D demonstrated reactions on the granular ER, the vesicles, the MVBs, and the secretion granules that were unaffected by ouabain. No reaction was seen on the Golgi apparatus or on the mitochondria, as had been observed throughout the Adriamycin-treated material. Finally, precipitate along the membranes of the cell was evident, especially when seen on cellular extensions within the osteodentin deposit. Again, no reaction was seen in direct association with either the pulpal or osteodentinal matrices.

The group E sections incubated in **medium 1** demonstrated enzyme activity similar to those of groups C and D, but with some notable differences. The granular ER demonstrated some reaction in the cells incubated for 30 minutes, but this was diminished in the 50 minute incubation sections. Reactions were seen on the vesicles and MVBs in sections from both incubation times, but the secretion granules showed the heaviest reaction in these same cells, especially in the sections incubated for 50 minutes. The coated vesicles also had some precipitate on their membranes. The Golgi apparatus demonstrated no reaction, identical to the observations above. Mitochondria again had no reaction on their membranes or cristae. The cellular membranes showed some reaction, and this could be seen in the extensions within the osteodentin deposit. As mentioned above, some background reaction was seen on the cells and the matrix in the

sections incubated for 50 minutes, but this did not obscure the reactions on any organelle.

The group E sections that were heat-inactivated and then incubated in **medium** 2 showed a greatly reduced or absent reaction (Figure 40). A very slight reaction was seen along the granular ER of the cells and over the matrix. The sections incubated in **medium** 3 from this same group demonstrated activity similar to other sections incubated in the presence of levamisole. The granular ER exhibited some reaction in the cells incubated for 30 minutes (Figure 41), but this reaction was very slight or absent in the sections incubated for 50 minutes (Figure 42). The Golgi apparatus showed no reaction, but the vesicles and MVBs had slight reactions along their membranes. The mitochondria of these odontoblast-like cells also exhibited no reaction precipitate (Figure 41). However, the secretion granules of the cells had a heavy reaction associated with them, especially when incubated for 50 minutes. These granules were distinct and generally were seen near the cell membranes and in cellular extensions (Figures 41-44a). They were also more evident in cells found within the deposit than in cells around the periphery (Figures 43, 44). There was a concentration of reaction along the membranes of the cells and their extensions (Figures 44, 45). Some reaction was seen in the osteodentin matrix but appeared to be associated with the membranes of structures or extracellular vesicles

(Figure 44b). Some of these reactions were seen over the full extent of the structure (Figure 44c). Finally, coated vesicles found within the cells and their extensions also exhibited some reaction precipitate (Figure 42).

Material from group E incubated in **medium 4** showed reactions that were very similar to those seen in the sections incubated in medium 1. The granular ER showed some reaction when incubated for 30 minutes, but almost none when incubated for 50 minutes. The vesicles, MVBs, and secretion granules all had a reaction associated with them, especially in the 50 minute incubated sections. The Golgi apparatus again had no reaction evident in any of the cells seen. The mitochondria also had no reaction along the membranes or cristae. The odontoblast-like cells had some reaction along the cellular membranes. Background reaction was noticed both on the cells and the matrices, pulpal and osteo-dentinal.

In summary, the reaction pattern seen in the sections 7 days after Adriamycin administration was similar in both groups C and D, and group E, even though different incubation media was used for the group E sections. Heat-treatment prior to incubation inactivated the enzyme activity seen in the odontoblast-like cells, with the exception of some slight precipitate seen on the granular ER. Reaction seen along the granular ER also was reduced or absent in sections incubated in the other media for the longer time periods

(50 and 60 minutes). Precipitate was seen in association with the vesicles, the MVBs, and the secretion granules, but only the reaction of the secretion granules was not diminished by the addition of levamisole. The Golgi apparatus and mitochondria demonstrated no reactivity in the odontoblast-like cells, regardless of the incubation media. The cellular membranes demonstrated some reaction, especially on cellular extensions within the osteodentin deposit. The addition of ouabain to the incubation media had no observed effect upon the observed alkaline phosphatase activity of the cells. Finally, any reaction seen in the osteodentin matrix was associated with cellular extensions or extracellular vesicles.

As stated above, the endothelial cells of the blood vessels in the pulp were unaffected by the Adriamycin treatments. Their enzyme activity was similarly unaffected, and the observations concerning their activity are identical to those of group B sections. Even those cells found within the osteodentin deposit exhibited no altered alkaline phosphatase activity. Thus, when incubated in **medium 1**, the granular ER, the vesicles and the cellular membranes demonstrated some reaction. This was the same as that seen in sections from **medium 4**. Sections incubated in **media 2 and 3** had diminished reactions. Very slight, if any reaction was present within the cells or along their limiting membranes. An observation to be noted was that mitochondria in these

drug-treated sections exhibited a reaction that was present in sections from media 1 and 3, and was absent in the presence of ouabain in medium 4.

A summary of this section is presented in Table 2.

Table 2

A summary of the Results section, detailing the observed reaction associated with the different cell types. The symbols represent arbitrary units of reaction intensity.

- \- No reaction
- \+ Slight reaction
- ++ Moderate reaction
- +++ Heavy reaction

(1)-Number of vesicles exhibiting activity reduced.
(2)-Not enough cells observed to define activity.
(3)-Reduced or absent activity in sections incubated longer than 30 minutes.
(4)-Cells observed only in the apical pulp in association with the osteodentin matrix.

CELL TYPE	MEDIUM 1			MEDIUM 2			MEDIUM 3			MEDIUM 4		
	AP.	MID.	IN.	AP.	MID.	IN.	AP.	MID.	IN.	AP.	MID.	IN.
Immature fibroblast	—			—			—			—		
Undifferentiated mesenchymal cell	—	—	—	—	—	—	—	—	—	—	—	—
Pulpal fibroblast												
granular ER	+	+	+	—	—	—	+	+	+	+	+	+
vesicles	+	+	+	—	—	—	+	+	+	+	+	+
Golgi apparatus	—	—	—	—	—	—	—	—	—	—	—	—
mitochondria	+	+	+	—	—	—	+	+	+	—	—	—
multivesicular bodies	+	+	+	—	—	—	+	+	+	+	+	+
cell membrane	+	+	+	—	—	—	—	—	—	+	+	+
Subodontoblastic fibroblast												
granular ER	++	++	++	—	—	—	++	++	++	++	++	++
vesiscles	++	++	++	—	—	—	+(1)	+(1)	+(1)	++	++	++
Golgi appartus	—	—	—	—	—	—	—	—	—	—	—	—
mitochondria	++	++	++	—	—	—	++	++	++	—	—	—
multivesicular bodies	++	++	++	—	—	—	++	++	++	++	++	++
cell membrane	+	+	+	—	—	—	—	—	—	+	+	+
Endothelial cell												
granular ER	+	+	(2)	—	—	(2)	+	+	(2)	+	+	(2)
vesicles	++	++		—	—		—	—		++	++	
Golgi apparatus	—	—		—	—		—	—		—	—	
mitochondria	++	++		—	—		++	++		—	—	
cell membrane (luminal and abluminal)	++	++		—	—		+	+		++	++	
Odontoblast-like cell (4)												
granular ER	+++(3)			+			+++			+++		
vesicles	+++			—			+++			+++		
Golgi apparatus	—			—			—			—		
secretion granules	+++			—			+++			+++		
mitochondria	—			—			—			—		
multivesicular bodies	+			—			+			+		
cell membrane	+++			—			++			+++		

DISCUSSION

Morphology of Normal Pulp Cells

The morphology of pulpal cells seen in this study confirms the observations of Han, Avery, and Hale (1967) concerning the pulp cells of rodent incisors. Others (Harris and Griffin 1967, 1969) have seen similar results in pulps from human teeth. The pulpal fibroblasts undergo maturation as they are carried incisally by the growth of the incisor. In the apical third, many of the fibroblasts were immature, and were not active as indicated by their lack of developed organelles. Some fibroblasts had developed organelles and were presumably responsible for the formation of the matrix components of this portion of the pulp. Within the middle third of the pulp, more cells and cellular extensions were present, indicating an increased cellular density. The fibroblasts were mature and active secretory cells, having profiles of granular ER and Golgi saccules. Vesicles were abundant, further indicating transport and/or secretion of materials was taking place. The matrix was more fibrillar, with some fibrils associated with the cells or cell membranes (Figure 3). In the incisal third of the pulp, mature secretory fibroblasts were seen, many of these closely associated with the fibrils of the matrix (Figure 5). The pulpal matrix became increasingly more fibrillar as one continued incisally. Increasing

numbers of older, inactive cells that resembled fibrocytes have been seen in the incisal third of rodent pulps (Han, Avery, and Hale 1965), but they were rarely observed in this study. Cells resembling these fibrocytes have recently been seen in vitro (Karim and Singh 1987). These cells contained a paucity of organelles, but many microtubules were found in their cytoplasm. They were mainly seen at about 28 to 30 days after the initial culturing of the rat pulp. The entire cellular population of the rat incisor is renewed in a time period of approximately 32 days (Smith and Warshawsky 1976). The pulpal fibroblasts may become inactive and lose their organelles, becoming fibrocytes, as they approach the end of their life cycle, which corresponds to the migration and renewal cycle of the rat incisor. Therefore, the rat incisor pulp contains fibroblasts in all stages of their life cycle, from differentiating cells in the apical pulp to inactive, dying fibrocytes in the incisal end.

The observation that different "subtypes" or phenotypes of pulpal fibroblasts were present throughout the pulp is not unique. Karim and Singh (1987) also have seen what may be morphologically distinct subpopulations of cells growing in cultures of rat incisor and human molar pulps. Additionally, Karim (1987), in a scanning electron microscopic study, observed cells having different surface features, lending support to this idea. Recent reports suggesting that 2 functionally heterogeneous subpopulations of gingival

fibroblasts may exist in culture (Hassell and Stanek III 1983). These 2 subpopulations may be responsible for the simultaneous production of types I and III collagen by cultured gingival fibroblasts (Engel et al 1980). Since both types I and III collagen has been demonstrated in the pulpal matrix *in vivo* (Magloire et al 1982; Linde 1985b), 2 distinct subpopulations, each synthesizing one type of collagen, may exist in the pulp. The other components of the pulpal matrix [noncollagenous proteins, fibronectin, lipids, etc. (Linde 1985b)] also may be produced by a separate subpopulation of pulpal fibroblasts. Clearly, more research is necessary to confirm the existence and function of these different cell types.

The cells of the cell-rich subodontoblastic zone (SO zone) have been previously studied in rat incisors (Gotjamanos 1969a), rat molars (Gotjamanos 1969c), monkey molars (Gotjamanos 1969b), and human teeth (Harris and Griffin 1969). Only Gotjamanos concentrated on the ultrastructure of the cells of this zone. He found cells having little granular ER and a poorly developed Golgi apparatus. These cells did possess mitochondria and cellular processes. They were unlike fibroblasts from the centre of the pulp, and the matrix surrounding them was lacking in collagen fibres (Gotjamanos 1969a). This is in contrast to the present study, which found the SO zone of the rat incisor to consist of undifferentiated mesenchymal cells and

fibroblasts that were further differentiated than those of the rest of the pulp. The latter cells had much granular ER, a highly developed Golgi apparatus, and cellular processes (Figures 6, 9, 13). These cells were quite active, as indicated by the dilation of the granular ER and the number of vesicles within the cell. Collagen fibrils also were observed, some in close association with the fibroblasts. Undifferentiated mesenchymal cells were observed as having few organelles or cytoplasmic extensions, but did contain some mitochondria. It is possible that Gotjamanos mistook these undifferentiated mesenchymal cells as being the only cell type found in the SO zone. The present results confirm those of Harris and Griffin (1969), who also observed both fibroblasts and undifferentiated mesenchymal cells in the cell-rich zone of human pulps.

The role of the pulpal fibroblasts of the SO zone has yet to be conclusively determined. The cells have a definite synthetic activity, but what they produce and secrete is not yet known. They have been implicated in the formation of the collagenous von Korff's fibres (Weidenreich 1925; Baume 1980; Wigglesworth et al 1986), and in the formation of type III collagen (Magloire et al 1982). They may aid in the transport of materials from the pulp proper to the odontoblast cell layer (Gotjamanos 1969a), or may play a role in supporting the function of the odontoblasts (Gotjamanos 1969b; Linde 1985b). The latter idea is supported by the

histochemical investigations by Goggins and Fullmer (1967) and others (Heyden and From 1969; Severson 1971). This is also supported by the high enzyme activity observed in association with these cells in this study. Some fibroblasts in the SO zone of human pulps may have differentiated into fusiform, bipolar cells. These cells, called Höhl's cells (Baume 1980), are thought to be important in the transmission of nervous impulses between the pulp and the odontoblast cell layer (Köling and Rask-Andersen 1983). Both the fibroblasts and the undifferentiated mesenchymal cells are thought to be reserve cells for the replacement of dead or damaged odontoblasts. Studies on the repair process of damaged pulpal tissues (Fitzgerald 1979; Yamamura 1985; Schröder 1985) have shown that damaged odontoblasts are replaced by cells that have migrated from the pulp and have undergone differentiation into odontoblasts. Authors have hinted that the origin of these cells is from the pulp and, more specifically, from the subadjacent layer to the odontoblasts, i.e., the subodontoblastic zone (Feit 1970; Fitzgerald 1979). This last proposal may be supported by some of the observations in this study. The fibroblasts in this zone are further differentiated than other pulp fibroblasts, and may only need a slight inducement to become odontoblasts or odontoblast-like. As well, undifferentiated mesenchymal cells that may be able to differentiate into any type of pulpal cell are concentrated in this zone.

Alkaline Phosphatase Activity of Normal Pulp

The non-specific alkaline phosphatase (APase) and the magnesium-, calcium-activated adenosine triphosphatase (Mg^{2+}-,Ca^{2+}-ATPase) activity observed in normal pulp cells in this study is similar to that seen by other investigators. As seen in Table 2, immature fibroblasts, primarily in the apical pulp, and undifferentiated mesenchymal cells throughout the pulp showed no reaction indicating enzyme activity. Inactive fibroblasts seen only in the incisal pulp were too few in number for a definite conclusion to be made regarding enzyme activity. Only the mature active fibroblasts of the pulp and the subodontoblastic (SO) zone and the endothelial cells of the pulpal blood vessels demonstrated sites of enzyme activity.

In general, the fibroblasts of the pulp showed minimal enzyme activity. APase activity, determined using the APase inhibitor levamisole, was limited to the cell membranes, while the remainder of the organelles showing the reaction (granular ER, vesicles, and multivesicular bodies) demonstrated Mg^{2+}-,Ca^{2+}-ATPase activity. The reaction associated with the mitochondria was absent when the cells were incubated in the presence of ouabain, indicating the presence of a Na^{+}-,K^{+}-ATPase on these organelles. The fibroblasts of the SO zone had a more intense reaction associated with the cellular organelles, indicating a higher level of enzyme activity. APase activity was again associated with the

cellular membranes, but some vesicles also showed a reaction. The mitochondrial reaction was again due to a Na^+-,K^+-ATPase, demonstrated by the inhibition of the reaction by ouabain. Thus, the reaction associated with the granular ER, a number of vesicles, and multivesicular bodies is due to Mg^{2+}-,Ca^{2+}-ATPase. These results imply that this ATPase activity in pulp fibroblasts is primarily associated with organelles of the secretory pathway: the granular ER, vesicles, and secretion granules (Figure 16). Although no reaction was visible on the Golgi saccules of the cells (Figures 6, 10), it may be present but not seen due to the level of sectioning of the material. As well, vesicles in the Golgi region that show a reaction (Figures 6, 10) may be derived from the Golgi saccules.

Most research dealing with the enzyme activity of dental pulp has been histochemical studies done at the light microscopic level. APase activity was found to be highest on the cells of the SO zone and the matrix between these cells (Harris 1950; Goggins and Fullmer 1967; Kiguel 1970; Magnusson et al 1974). Fibroblasts in the remainder of the pulp had no activity associated with them. The few studies done at the electron microscopic level (Yoshiki and Kurahashi 1971; Leonard and Provenza 1973) have shown the reaction to be associated with the cell membranes and the intercellular matrix in the SO zone. Activity has also been observed on the membranes of vesicles within the cells (Yoshiki and

Kurahashi 1971; Lunt and Noble 1972). The present study has confirmed the localization of the enzyme to the cell membranes and vesicles, but at no time was any reaction seen on the pulpal matrix, either in the central pulp or in the SO zone. Results of biochemical studies involving pulp cells also support this localization. Messer et al (1978) found APase activity of the central pulp to be less than that of the periphery, i.e., the SO zone. They also found that the greatest activity was in the plasma membrane fraction. This was confirmed by Guo and Messer (1978) who also found that APase was a membrane bound enzyme. This latter fact indicates that the enzyme may be found on vesicular membranes within the cell, as seen in this study.

The localization of Mg^{2+}-,Ca^{2+}-ATPase in cells of the pulp has also been extensively studied. Light microscopic histochemistry has shown the central pulp cells to have little activity, but the cells of the SO zone have a much greater enzyme activity (Heyden and From 1967; Severson 1971). Ultrastructural localization has been accomplished through biochemical rather that electron microscopic means. Mg^{2+}-,Ca^{2+}-ATPase activity has been found in the microsome fraction (Yoshimura and Suzuki 1975; Abiko 1977; LeBell 1981) and the plasma membrane fraction (Guo and Messer 1976, 1978; Messer et al 1978) of homogenized and fractionated dental pulps. These studies have also shown that the enzyme is membrane-bound, and thus it may be associated with the

endoplasmic reticulum, vesicles, Golgi sacculles, multivesicular bodies, and cell membranes. However, all of the above biochemical studies used pulps that included the odontoblasts, which have been shown to exhibit Mg^{2+}-,Ca^{2+}-ATPase activity on cellular membranes (Heyden and From 1967; Granström and Linde 1974, 1976; Magnusson and Linde 1974) and vesicles within the cells (Granström et al 1978; Linde and Granström 1978). No study isolated the odontoblasts from the remainder of the pulpal cells. Therefore, the localization of the enzyme to organelles that make up the microsome fraction seen in the present study confirms the observations of others.

The significance of the presence of both of the enzymes in pulpal cells is undetermined. Most of the functions attributed to "alkaline phosphatase" were in studies performed previous to the recognition of the enzyme Mg^{2+}-,Ca^{2+}-ATPase, and therefore, many of these same functions are presently being attributed to the latter, more specific enzyme. The non-specific alkaline phosphatase (APase) has many possible functions in association with mineralizing tissues, but few when seen on non-mineralizing cells. The enzyme is known to hydrolyze phosphate esters and liberate inorganic phosphate (Pi) (Robison 1923). This increases the local concentration of Pi, which may be used in a number of ways by the non-mineralizing cell. The cell may bind the Pi to its cell membrane, or to receptors on this membrane which

may act to regulate cellular functions (Shapiro and Golub 1981), or it may act to regulate cell differentiation or division (Moog 1944, 1965; McWhinnie and Saunders 1966; Wuthier and Register 1985). This latter function has recently been disputed by Whyte and Vrabel (1987). Both these functions are possible when pulp cells are considered, but only the former can be supported by the present study. No enzyme activity was evident on immature or differentiating pulp cells. Only the mature pulpal fibroblasts, whose functions certainly may have been regulated by the enzyme, demonstrated activity. The most likely function for the enzyme in pulp fibroblasts is the transport of substances. This has been postulated as a function of APases associated with intestinal cells (Hugon and Borgers 1967, 1968; Hugon 1970), kidney tubule cells (Mölbert et al 1960), endothelial cells (Clark 1961; DeThé 1968), and cells of the placental membranes (Manning et al 1966). This has also been a suggested function of the enzyme when seen on stellate reticulum cells of the developing human teeth (Lunt and Noble 1972). These substances, probably metabolites, may originate from the pulpal blood vessels, whose cells also exhibit APase activity. In the present study, it was primarily seen on the luminal and abluminal membranes and vesicles (Figures 19-31, Table 2). This may indicate that the substances are transferred across the luminal membrane

to vesicles which deliver the substances to the abluminal membranes. They are then taken up by the pulp cells and employed in metabolism or synthesis, or they may be transferred to other cells. This sequence parallels that set out by Kiguel (1970). The increased activity of the fibroblasts of the SO zone could thus be explained. These cells may be transporting materials from the subodontoblastic blood vessels to the odontoblasts, where they may be involved in metabolic processes (Gotjamanos 1969a). The substances may be phosphorylated to aid in their transfer by either the APase or an enzyme complex incorporating the APase. Lipids (Madsen and Tuba 1953), amino acids (Trintophyllopoulus and Tuba 1959), and glucose (Ten Cate 1962; Linscheer et al 1967) have been suggested as being some of the transferred metabolites. The enzyme function may actually be a combination of these, as no one theory is adequate to explain the role of APase in pulp.

In contrast to the role of APase in pulp cells, the role of Mg^{2+}-,Ca^{2+}-ATPase is more defined. Although the latter enzyme may be of more importance in the mineralization of tissues, its function in the pulp has not been overlooked. Most studies concerning this enzyme in the pulp associate it with intracellular calcium transport (Guo and Messer 1976; Abiko 1977; Messer et al 1978; Messer and Guo 1979; LeBell 1981). The enzyme would act as a "calcium pump" (Anderson 1973), accumulating calcium from the cell

and "packaging" it in vesicles. The ATP would act as a source of energy for this pump (Anderson 1973), or as others have suggested, as a source of Pi that may also be found in the vesicles (Messer and Guo 1979). These vesicles may then be transferred, from the pulp fibroblasts to the SO zone, and from the SO zone cells to the matrix or odontoblasts. The localization of the enzyme on the components of the secretory pathway of the fibroblasts in this study would confirm this proposed function. That the enzyme activity is higher in the cells of the SO zone can also be explained. These cells would transfer greater amounts of calcium to the odontoblasts to aid in their formation of the dentinal matrix. This function has been proposed for many other cells demonstrating ATPase activity. Intestinal mucosa (Melancon and DeLuca 1970; DeLuca and Schnoes 1976), kidney tubule cells (Parkinson and Radde 1971; Rorive and Kleinzeller 1974), erythrocytes (Schatzman and Vincenzi 1969; Schatzman and Rossi 1971), and placental membrane cells (Shami and Radde 1971) carry out active transport of cations across their membranes employing specific ATPases using energy derived from ATP.

The enzyme associated with the multivesicular bodies in the fibroblasts of the pulp probably does not share the functions outlined above. It is known that multivesicular bodies (MVBs) are forms of lysosomes that contain a number of vesiculated hydrolytic enzymes that are responsible for

the breakdown of ingested particles, collagen fibres, etc. (Alberts et al 1983). Not all the enzymes within these lysosomes have yet been characterized (Alberts et al 1983). The presence of an Mg^{2+}-,Ca^{2+}-ATPase or similar ATPase in the MVBs may be valid, since the reaction seen on these organelles was a consistent finding in the fibroblasts of the pulp and the SO zone. Additional investigation is necessary to confirm this idea.

Effect of Adriamycin on Pulp

Adriamycin (doxorubicin hydrochloride) is an anthracycline antibiotic, isolated from cultures of **streptomyces peucetius caesius.** It has been in use as an antineoplastic agent since the early 1970s. Its antineoplastic capabilities are probably due to its ability to intercalate itself between adjacent base pairs in the double helical structure of DNA molecules (DiMarco 1975). Both DNA and RNA synthesis is halted after uptake of the drug by the cell (DiMarco 1975). The drug is not phase specific, but is most lethal in the late G_1 or S phase of the cell cycle (Bhuyan et al 1980), the period in which the greatest amount of DNA synthesis is taking place. The actual death of the Adriamycin-treated cell is probably due to the conversion of the drug to free radicals by the microsomes of the cell (endoplasmic reticulum, Golgi apparatus, etc.). These free radicals cause membrane lipid peroxidation or DNA breakage,

which leads to cell death (Bachur et al 1978). Adriamycin is one of the most useful and most effective cytotoxic antineoplastic drugs. It has major clinical applications in carcinomas of the breast, endometrium, ovary, lung, and in the treatment of many sarcomas, including neuroblastoma, rhabdomyosarcoma, and in particular, childhood osteosarcomas. It is also effective against leukemias and other hematologic cancers (Salmon and Sartorelli 1984).

The level of Adriamycin used in this study, a single dose of 5.0 mg/kg, has been used previously to induce osteodentin formation in rats (Dahl 1984; Karim 1985b). It is also a dose consistent with therapeutic results in experimental trials in rats (DiMarco 1975). The effects of Adriamycin on the pulp of the rat incisor can be seen as early as one hour following administration of the drug (Karim, unpublished results), and have been documented one day after the drug treatment (Karim and Pylypas 1985; Karim 1985a). The cells of the pulp were affected in different ways. The early preodontoblasts and their precursor cells were killed and underwent necrosis (Dahl 1984; Karim 1985a, b). These proliferating cells were undergoing cell division and may have been in the late G_1 or S phase of their cell cycle (Dahl 1984; Karim and Pylypas 1985). The late pre-odontoblasts and young odontoblasts that had differentiated to the stage of initial dentin production were seemingly unaffected by the drug (Karim and Pylypas 1985; Karim

1985a). No other areas of the pulp appeared to be affected by the drug at this time.

Three days following administration of the Adriamycin, numerous changes were seen in the pulp. Some necrotic debris remained in the area of the preodontoblasts and their precursors. Karim (1985b) has seen aggregations of mesenchymal cells in the pulp and subodotoblastic zones just incisal to these necrotic areas. These cells were also associated with an increased number of collagen fibres in the matrix (Karim 1985b). This observation was confirmed by this study, which showed the initiation of the osteodentin matrix (Figures 25-28) was found in the area of the subodontoblastic zone of the apical pulp incisal to the lesion. Processes of these odontoblast-like cells were closely associated with this matrix. Secretion granules and microfilaments were found within these processes, indicating some connection between the cells and the osteodentin matrix formation. The matrix itself contained irregularly arranged collagen fibres, and areas of fibrils and microfilaments within as well as outside these cellular processes. This tissue closely resembled the matrix of mantle dentin, although matrix vesicles associated with mantle dentin production and mineralization (Bonucci 1984) were not present.

Five days following Adriamycin treatment, the number of odontoblast-like cells had increased. They were found on

the periphery and within the osteodentin matrix, which had also increased in size. No necrotic cells were seen, but degenerating cells were present within the osteodentin. The condition of these latter cells may be due to a delayed toxicity of the drug. These cells may have been affected by Adriamycin initially, but did not die immediately. The odontoblast-like cells were highly active, indicated by the abundant profiles of dilated granular ER and secretion granules. The osteodentin matrix consisted of collagen fibres oriented in many different planes with large amounts of amorphous ground substance between them (Figure 30). The matrix appeared less dense than the dentinal matrix, probably due to this large amount of ground substance (Takuma et al 1968). This prompted Dahl (1984) to call the osteodentin matrix irregular predentin tissue, although no tubules were observed in the tissue. Mineralized matrix was also seen in these sections (Figure 29). These crystals were unlike those of dentin or enamel and had no apparent ordered arrangement. Therefore, 2 portions of the osteodentin deposit were observed. The periphery was an unmineralized collagenous matrix, and the central portion was a more dense, mineralized matrix. Both of these matrices contained cellular inclusions and processes, but, unlike dentin, were atubular. These two tissues have been termed preosteodentin and osteodentin, respectively (Karim and Pylypas 1986).

Seven days following drug administration, the osteodentin deposit was larger and was located more incisally than that seen at three days after treatment. This incisal migration is due to the continuous eruption of the rat incisor, which is unaffected by Adriamycin treatment (Karim, in preparation). Many odontoblast-like cells were associated with this deposit, both around the periphery and within the matrix. The characteristics of both the cells and the matrix was similar to that seen in the sections five days after the drug treatment.

The presence of odontoblast-like cells in sections three days following Adriamycin administration indicated that some of the cells that had not undergone necrosis in previous studies of Adriamycin-treated pulps at one day (Dahl 1984; Karim 1985a) were indeed affected. Which cells were affected and how they were induced to differentiate are as yet unanswered questions. Dahl (1984) believed "pulp cells adjacent to the preodontoblastic zone...as well as further into the pulp were converted" to form odontoblast-like cells. Karim (1985a) observed that the mesenchymal cells incisal to the lesion aggregated on the lateral and mesial walls of the pulp chamber prior to the formation of osteodentin at the same sites. It has also been seen that cells just incisal to the initial lesion take up abnormally high amounts of ^{3}H-thymidine one day following Adriamycin administration (Karim, in preparation). These labelled

cells were located in the subodontoblastic zone and the pulp adjacent to that area, and were eventually observed associated with the osteodentin matrix seven days later (Karim, in preparation) These results indicate that the odontoblast-like cells were derived from pulp cells that underwent induced differentiation. This is supported by the work of Stene (1979) with vincristine, Nogueira et al (1981) with colchicine, and Koppang (1981) with cyclophosphamide who all found that abnormally differentiated pulp cells formed an osteodentinal matrix following drug administration. This process has also been seen in pulps following cavity preparation. Fitzgerald (1979) showed that ^{3}H-thymidine-labelled pulp cells differentiated into odontoblasts following damage to the odontoblast layer. Furthermore, his results implied that these cells came mainly from the the subodontoblastic zone of the tooth. Yamamura (1985) also found that the cells of the pulp differentiated into odontoblasts to replace the ones lost to the cavity preparation procedure. Using ^{3}H-thymidine labelling and radioautography, it was observed that undifferentiated mesenchymal cells could differentiate into functioning odontoblasts in less than four days. The odontoblast-like cells in our study were seen three days after the Adriamycin administration, and they were associated with the initiation of the osteodentin matrix.

Other studies have postulated that the origin of the odontoblast-like cells were altered odontoblasts. Stene and Koppang (1980) believed that vincristine-altered odontoblasts were responsible for the production of an osteodentinal matrix. Orams (1983) thought depolarized odontoblasts laid down an irregular matrix after cyclophosphamide administration. Dahl and Koppang (1985) believed that a portion of the osteodentin matrix was also produced by depolarized odontoblasts after Adriamycin administration. Recent evidence disputes this theory. Karim (in preparation) has seen a layer of transformed cells from the subodontoblastic zone that were situated between the periphery of the deposit and the odontoblast layer. There was little evidence of depolarization of the odontoblasts, nor of their involvement in the production of the osteodentin matrix.

The initial inducement of differentiation of the odontoblast-like cells is as yet unknown. The differentiation of the dental papilla into odontoblasts in the normal development of the tooth requires the presence of the internal enamel epithelium and the basal lamina (Ruch 1985). A number of interactions between the two cell types takes place prior to differentiation and proper functioning of both derivative cell types (Ruch 1985). No basal lamina nor cells that resemble those of the internal enamel epithelium were seen in the drug treated pulps. The differentiation of pulp cells into odontoblasts to replace those damaged by

dental procedures may be the result of a number of factors. Yamamura (1985) speculated that the inducing agents may be dentin chips left from the procedure, the microenvironment of the pulp, or calcium hydroxide pulp capping agents. Rather than the calcium hydroxide being the inducing agent, Schröder (1985) believed that the necrosis caused by the pulp capping agent was the stimulus for differentiation. This was supported by Veis in 1985. This idea was initially employed by Koppang (1973) to explain the production of an osteodentinal matrix in rat incisors after the administration of cyclophosphamide. The cell necrosis supposedly induced the pulp cells to differentiate and produce the matrix, as a protective or reparative phenomenon. Dahl (1985, and with Koppang 1985) used this same explanation when Adriamycin was used. If this is true, then all pulp cells that come in contact with necrotic debris should become odontoblast-like and produce osteodentin. However, this was not seen in any studies. Karim and Pylypas (1985) determined that after a single dose of Adriamycin, a necrotic lesion extended for approximately 2.2 mm from the apical end of the rat incisor, and only on the periphery of the pulp chamber. If the necrotic debris were responsible for cell differentiation and the subsequent formation of the deposit, the osteodentin would be observed pulpal to and along the length of this lesion. However, osteodentin deposits have

only been seen along the mesial and lateral walls of the pulp chamber and incisal to the site of the lesion (Karim 1985b). Furthermore, as stated above, only the cells incisal to the lesion demonstrated increased ^{3}H-thymidine labelling one day after drug treatment (Karim, in preparation). This evidence shows that another explanation is needed to explain the inducement of pulp cell differentiation. Adriamycin itself may be the inducing agent. The drug is known to be toxic to cells in the late G_1 or S phase of the cell cycle (Bhuyan et al 1980), and it was shown to act at the nuclear level (DiMarco et al 1971). It may also cause aberrations to cells of the pulp that are not cycling. Dahl and Koppang (1985) believed a direct but non-lethal injury to odontoblasts may have caused them to depolarize and secrete osteodentin. This drug may act similar to forskolin, which was found to induce the differentiation of newborn mouse calvaria cells into functioning osteoblasts (Hakeda et al 1985). Formocresol may also possess this capability as it induces osteodentin production in pulps of human teeth (Keszler and Dominguez 1984), but further information is lacking. Additional research into Adriamycin's ability to induce differentiation is necessary to determine if the drug is indeed the inducing agent.

The amount of osteodentin matrix increased over the seven days of observation time, as did the number of odontoblast-like cells associated with it. Karim and Pylypas (1986) have shown that these cells secrete a proline-containing component of the osteodentin matrix which may be associated with collagen synthesis and secretion. The results in this study, especially those seen at three days after drug treatment, support the idea that the odontoblast-like cells are responsible for the formation of the osteodentin matrix. How the cell numbers increased is unknown. Dahl and Koppang (1985) observed that the odontoblast-like cells lack a proliferative capability similar to odontoblasts, and therefore do not multiply. The inducing effect of Adriamycin can only last as long as the drug is in the tissues, which is from 3-7 days after administration (Adria Laboratories report). The actual osteodentin matrix may play a role in the recruitment of additional odontoblast-like cells, through cell-matrix interactions. Many of these cells that were seen on the periphery of the deposit may have been pulp cells that, when in contact with the matrix, became odontoblast-like and secreted osteodentin. These cells would then become entrapped in the matrix that they were secreting, in a manner similar to that of osteoblasts in bone. This type of cell-matrix interaction has been documented (Ruch 1985; Yamamura 1985). Another explanation may be through cell-to-cell interactions whereby the

odontoblast-like cells may possess surface components that may act as inducing agents when they come in contact with receptors on pulp cell surfaces. More research is necessary to determine how the cell numbers are increased.

Using all of the above information, a detailed sequence of events concerning the formation of osteodentin in rat incisors following Adriamycin administration can be developed. Cells of the basal pulp and preodontoblast region are killed and a necrotic lesion is formed within 24 hours of drug treatment. Induced differentiation and aggregation of pulpal cells (probably fibroblasts and undifferentiated mesenchymal cells of the subodontoblastic cell-rich zone) follows. The synthesis and secretion of components of the osteodentin matrix by these newly differentiated odontoblast-like cells is initiated three days after the drug treatment. The mineralization and maintenance of this osteodentin deposit is the final step in this sequence. Odontoblast-like cells are added and the deposit grows, eventually to completely occlude the entire pulp chamber (Karim and Eddy 1984). This sequence is an expansion of that proposed by Karim (1985b) which paralleled the sequence developed by Bernick (1966) concerning osteodentin production in guinea pigs.

Alkaline Phosphatase Activity of Adriamycin-treated Pulp Cells

As presented in the results, the cells associated with the osteodentin matrix, the odontoblast-like cells, exhibited an altered alkaline phosphatase activity to that seen in untreated pulp cells. The pulpal fibroblasts in the treated sections demonstrated activity that was the same as that seen in the untreated or normal pulpal material. However, in the area of the developing osteodentin deposit (in the apical pulp), very few or no subodontoblastic fibroblasts were observed. This was especially evident in the sections from the pulps five and seven days following Adriamycin administration. The odontoblast-like cells on the periphery of the osteodentin were immediately adjacent to the odontoblast layer, with no other cells present between the two cell types. This has also been observed by Karim (in preparation). The sites of activity of these odontoblast-like cells closely resembled those of other published reports concerning odontoblasts.

The localization of APase activity in the odontoblast-like cells was limited to vesicles and to the cell membranes. When incubated in medium 3, which contained levamisole, an inhibitor specific for APase, the reaction on the cell membranes was reduced, and a smaller number of vesicles showing reaction were present. This corresponds to results that other researchers have found regarding odontoblasts.

Most studies have found the enzyme on the cell membranes (Harris 1950; Yoshiki and Kurahashi 1971; Leonard and Provenza 1973; Larsson 1973; Takano et al 1986) but some have seen it associated with vesicles within the odontoblasts (Yoshiki and Kurahasi 1971; Granström et al 1978; Granström and Linde 1981; Granström 1984). Some reports also have shown APase activity on extracellular vesicles and collagen fibres within the predentin matrix (Larsson 1973; Orams and Snibson 1982; Takano et al 1986). In the present study, APase activity within the matrix was associated with cellular membranes (Figures 26, 28), but not with components of the matrix.

The Mg^{2+}-,Ca^{2+}-ATPase localization in the odontoblast-like cells was also similar to published reports concerning odontoblastic enzyme activity. Organelles of the secretory pathway (the granular ER, vesicles, and secretion granules) demonstrated moderate to heavy reactions. Although no reaction was visible in association with the Golgi apparatus, it cannot be ruled out as a site for enzyme activity. The lack of observed reaction may be due to the level of sectioning, i.e., the saccules demonstrating the activity were cut so as to resemble vesicles. The cell membranes and multivesicular bodies (MVBs) also exhibited some enzyme activity that was not inhibited by levamisole. These results were consistent throughout the seven day observation period, i.e., the odontoblast-like cells three days after

Adriamycin treatment demonstrated the same sites of activity as those five and seven days after the treatment. The only difference observed was in the number of secretion granules visible in the odontoblast-like cells. The number of granules was greater in the seven day sections than in the three day or the five day sections. This may be related to the increased size of the osteodentin deposit or the increased mineralization of the matrix. The use of two different sets of incubation media (See Materials and Methods) also did not make a difference in the localization of the enzyme. Rather, the results of the sections from group E acted as a confirmation of the observations made on groups C and D. However, the increased time of incubation produced some unique results. The reaction intensity on most enzyme-active organelles was increased, but the reaction on the granular ER was decreased or absent in the groups incubated longer than 30 minutes (Figures 39, 42). It is unknown what may have caused this to occur, but we speculate that it may be due to inactivation of the enzyme on the granular ER through the increased exposure to the ingredients of the media, or through the depletion of activators (Mg^{2+} and Ca^{2+} ions) in the incubation media.

The above results agree with other reports of localization of Mg^{2+}-,Ca^{2+}-ATPase in odontoblasts. Severson (1968, 1971) showed that the activity of the odontoblasts was found

on the cell membranes. This was confirmed by Messer et al (1978) using biochemical means. Enzyme activity was demonstrated within the tooth germ by Yoshimura and Suzuki (1975), specifically in the microsome fraction consisting of endoplasmic reticulum and Golgi body membranes. Granström and Linde, in a number of papers, proved the existence of the ATPase in odontoblasts (Granström and Linde 1976, 1977), and also localized it to vesicles in the Golgi region using histochemical (Granström et al 1978; Linde and Granström 1978) and biochemical means (Granström and Linde 1981; Granström 1984). They did not observe any activity on the MVBs within the odontoblasts using histochemistry (Granström et al 1978), but the activity from the microsomal fractions may be in part from the MVBs. This activity associated with the MVBs is probably due to the presence of hydrolytic ATPases in these structures, as was discussed previously with respect to non-mineralizing cells. Granström et al also found no activity on the cell membranes, histochemically nor biochemically, although in the present study, using the same incubation media, some activity was seen (Figure 39). This may be explained through the process of exocytosis of the contents of the secretion granules and vesicles, whereby their membranes would be absorbed by the limiting membranes of the cell. The enzyme would be localized to these membranes, although it may not have a specific function. Some of the membrane material is resorbed by coated vesicles to

maintain celluar membrane structure (Alberts et al 1983), and thus, the reaction of the cell membranes and coated vesicles may be an artifact due to normal cellular processes rather than an indication of enzyme activity. Another interesting point is that Takano et al (1986) observed no ATPase activity on odontoblasts, yet this study, employing the incubation media described by them (with slight modifications), has found the activity to be quite distinct in association with odontoblast-like cells. Other investigators have found ATPases on other hard tissue-producing cells, such as ameloblasts (Crenshaw and Takano 1982; Takano et al 1986; Sasaki and Garant 1986) and cementoblasts (Granström 1982), but not in the same subcellular sites as in the odontoblast-like cells.

A number of cells found within the matrix were seen to be degenerating, probably through a delayed action of Adriamycin. These cells also possessed some alkaline phosphatase activity, although it was not determined if it was APase or Mg^{2+}-,Ca^{2+}-ATPase. The reaction was restricted to the vacuoles within the cell and the cell membranes. This is consistent with the results of Orams (1983). The degenerating cells in his study were within an irregular, osteodentinal-type matrix following cyclophosphamide administration. No explanation for this activity was offered by Orams, but we believe that it may be related to the presence

of a form of alkaline phosphatase in the lysosomes or MVBs of these cells, which would become quite evident during autolysis of the cells.

Background reaction was evident in the sections incubated for longer than 30 minutes (Figures 39, 42-45), and sometimes was present after only 30 minutes of incubation (Figure 41). This reaction was widespread throughout the cytoplasm of the cells, but was not specific for any organelle. This background was also present over the matrix in sections from group E (incubated in the media of Takano et al 1986), but was not observed in those from group D (media of Granström et al 1978). The background stain in the matrix was again not specific for any structure. Areas of heavy reaction were presumed to be cell process within the matrix. The activity was either associated with the secretion granules and the cell membranes of the process (Figures 43, 44a, c) or with the cell membranes alone (Figure 44b, 45). Another interesting observation was that the background reaction in sections from group E that were incubated in the presence of levamisole was less than that seen in sections from group D incubated in the absence of levamisole. Although the incubation time was longer for the group D sections, the excess reaction may be due to a low level activity of APase in the cytoplasm of the cells.

Heat inactivation of the enzyme prior to incubation in medium 2 was not as effective as that seen in the control

specimens. Some reaction precipitate was seen on the granular ER of some odontoblast-like cells, especially after incubation for longer than 30 minutes (Figure 40). This may indicate activity of an enzyme that is not heat labile, but no substrate (ATP) was in the incubation media. This precipitate was most likely due to an adherence of the metal ions of the media to the nucleic acids of the ribosomes, which was also observed by Granström et al (1978).

The activity associated with mitochondrial membranes seen in normal pulpal fibroblasts was not present in the odontoblast-like cells. Mitochondria in fibroblasts of the drug-treated pulps exhibited activity similar to that of the control cells, but no activity was seen on mitochondria in odontoblast-like cells, regardless of the incubation media used. This activity was present in odontoblasts in the study by Granström et al (1978), and was inhibited by the presence of ouabain in the reaction medium, indicating that the activity was due to a Na^+-,K^+-ATPase. This absence of activity may be due to the direct inhibition of the enzyme by Adriamycin (Gosalvez et al 1979) or the effect of the drug upon mitochondrial function (Ferrero et al 1976; Combs et al 1977) which may in turn inhibit the enzyme. A metabolite of the drug may be taken up by these cells, which would be susceptible to its effects (possibly cells at the G_2 stage in the cell cycle), and it may directly affect the mitochondria and their functions.

It has been known for some time that chelating agents, especially those used as demineralizing chemicals, can have an inhibitory effect upon alkaline phosphatases. EDTA and EGTA are two commonly used chelators that have been shown to inhibit both the APase (Magnusson et al 1974; Magnusson and Linde 1974; Granström and Linde 1976, 1977; Shapiro and Golub 1981) and Mg^{2+}-,Ca^{2+}-ATPase of odontoblasts (Magnusson and Linde 1974; Granström and Linde 1976, 1977; Granström 1984). It is believed that the chelating effect removes the cations necessary for the activation of the ATPase (Mg^{2+} and Ca^{2+} and for the proper function of the APase. EDTA has also been found to bind directly to the enzyme-substrate complex of APase (Wallace and Ko 1964) and may inhibit it further. The present study has shown that some inhibition is seen in sections demineralized prior to incubation (Figures 33, 37). The reaction is noticeably less intense than that seen in other sections incubated with specific inhibitors (Figures 34, 35). Even after reactivation in a solution containing Mg^{2+} ions, the enzyme activity was not as intense as previously observed.

Although the material in group E was demineralized using EDTA and was not reactivated, the enzyme activity from both incubation times was quite distinct. These results suggest that if the EDTA had an effect upon the enzymes,

their activity was very high in these cells prior to demineralization. In the original investigation (Takano et al 1986), the same methods (EDTA demineralization followed by incubation) yielded very distinct results demonstrating ATPase activity on ameloblasts. However, they were unable to observe any reaction on the odontoblasts. This may indicate that the ATPase of ameloblasts is resistant to EDTA inhibition, which is consistent with results of other researchers (Magnusson et al 1974; Magnusson and Linde 1974). The slight modificaton made in the present study (the lowering of the pH of the media in order to avoid precipitation) may have played a role in the retained activity on the odontoblast-like cells. It is possible that the Mg^{2+}-,Ca^{2+}-ATPase of these cells possessed optimum activity at a slightly lower but still alkaline pH value.

Significance of Altered Alkaline Phosphatase Activity

The alkaline phosphatase activity of the odontoblast-like cells is significant in that it is an indication that these cells may be involved in the mineralization of the osteodentin matrix. This also is an indication that their precursor cells, probably cells of the subodotoblastic zone, have changed from non-mineralizing tissue cells to those capable of the the production and subsequent calcification of a hard tissue matrix. The results of this study also

help support some of the theories regarding alkaline phosphatase, or more specifically APase and Mg^{2+}-,Ca^{2+}-ATPase, and mineralization.

The role of APase in the mineralization of tissues has yet to be conclusively determined, although several possible actions have been proposed. The hydrolysis of phosphate esters to liberate inorganic phosphate (Pi) and thus increase the local Pi concentration at calcification sites was the first role attributed to alkaline phosphatase (Robison 1923). It is believed that once the Pi concentration reaches a certain level, it contributes to the mineralization process through the formation of hydroxyapatite crystals. Most of the research on this role of the enzyme concerns matrix vesicle-mediated mineralization whereby the membrane-bound enzyme breaks down the phosphate esters to prepare the tissue for calcification. The matrix vesicle becomes the nucleating site for the initial crystal formation (Ali et al 1970; Majeska and Wuthier 1975). Although no matrix vesicles were observed in the osteodentin tissue in the present study, the activity of APase on the cell membranes indicate that this may indeed be a role of the enzyme when the odontoblast-like cells are near the mineralization front. The tissue immediately surrounding these cells would become mineralized and the cells would be trapped, as seen in this study.

Another possible role for the APase of the odontoblast-like cells may be to hydrolyze phosphate esters that act as crystal growth inhibitors. This theory, put forward by Neuman et al in a series of papers (1951, 1958, 1960, 1961; Fleisch 1964) states that APase may break down certain phosphate esters, notably ATP and inorganic pyrophosphate (PPi), that retard or inhibit the growth of hydroxyapatite crystals and the subsequent mineralization of the tissues. The odontoblast-like cells may employ this action to eliminate the inhibitors while also increasing the local Pi concentration to aid in the crystal formation of the osteodentin matrix.

The APase enzyme has also been proposed as a component in the regulation of cell differentiation or cell function (Shapiro and Golub 1981; Wuthier and Register 1985). As mentioned previously with the non-mineralizing pulp cells, little evidence was found in this study to either support or oppose this theory, although no hard tissue-producing cells observed undergoing differentiation had greater APase activities than those already differentiated.

Recently, a new role for alkaline phosphatase has been postulated. A glycoprotein that exhibits alkaline phosphatase activity and binds Ca^{2+} ions has been discovered in cartilage matrix vesicles (de Bernard et al 1985, 1986). It is unknown if this "new" enzyme is an APase or Mg^{2+}-,Ca^{2+}-ATPase, but it is inhibited by p-bromolevamisole, a specific

inhibitor of APase (de Bernard et al 1985). This new role, as a Ca^{2+}-binding protein, when combined with a role postulated by Reith (1983), may help shed light on the action of APase in mineralized tissue. Reith stated that calcium may be bound to certain carriers intrinsic to the limiting membranes of mineralized tissue-producing cells, specifically ameloblasts, and transported from one end of the cell to the area at which calcification is taking place. Since de Bernard's "new" APase is membrane bound (de Bernard et al 1985), it may be the carrier that Reith proposed. As well, APase can be found on the membranes of most hard tissue cells. Thus this new role of APase may be transcellular calcium transport, from one end of the cell, where it is taken up and bound to this enzyme, to the other, where mineralization is taking place. Therefore, the odontoblast-like cells, exhibiting some APase activity along their membranes, may employ this function to aid in the mineralization of the osteodentin matrix.

In contrast to APase, the role of Mg^{2+}-,Ca^{2+}-ATPase in the mineralization of tissues, especially dental tissues, is more defined. As previously discussed, this enzyme is thought to act as a "calcium pump" which would operate to increase the concentration of calcium within subcellular or extracellular structures of mineralizing tissues (Anderson 1973; Granström and Linde 1981; Sasaki and Garant 1986).

Much of the investigation into this function of the enzyme has involved extracellular matrix vesicles (Anderson 1973; Larsson 1973; Bonucci 1984). Calcium ions may be transported across the membranes of vesicles by these enzymes that would be dependent upon ATP for their energy (Ali and Evans 1973; Väänänen 1980). The accumulation of Ca^{2+} ions would reach a level where, along with the increased Pi levels contributed by APase, hydroxyapatite crystals would form. The increase in size of these crystals would cause the membraned vesicles to rupture, and the crystals, exposed to the extracellular matrix which has naturally high levels of calcium and phosphate, would continue to grow to their normal length (Anderson 1973).

An ATPase having the same qualities as this calcium pump has also been associated with secretory ameloblasts. It has been found on the cell membranes and Tomes processes (Crenshaw and Takano 1982; Sasaki and Garant 1986) and within the Golgi cisternae and secretory vesicles of these cells (Takano et al 1986). Crenshaw and Takano (1982) believe this pump is responsible for the regulation of calcium transport through secretory ameloblasts to the developing enamel matrix.

The recognition of a Mg^{2+}-,Ca^{2+}-ATPase in odontoblasts was mainly due to the work of Granström et al. In a series of papers (1976, 1977, 1978, 1979; Linde 1981; Granström 1984), these authors discovered and characterized this

enzyme. As discussed previously, they also localized it within odontoblasts using histochemical and biochemical techniques. The role of the Mg^{2+}-,Ca^{2+}-ATPase in odontoblasts, according to them, is transmembraneous transport of Ca^{2+} ions (Granström and Linde 1978). Odontoblastic intracellular vesicles demonstrating enzyme activity were found to have an ATP-dependent uptake of Ca^{2+} ions, which corresponds to the proposed action of the enzyme on these structures (Granström and Linde 1981). Further evidence has shown that the enzyme, along with APase, may have two actions within the odontoblasts. Two populations of vesicles may be present in the cell, "one involved in intracellular Ca^{2+} homeostasis and the other in promoting extracellular mineralization" (Granström 1984). Linde has further speculated that the role of the enzyme in calcification may be one of two possibilities. The intracellular vesicles may accumulate and concentrate the Ca^{2+} ions, and then transport them to the mineralization front, where they would be used in the formation of hydroxyapatite crystals. The second possible role would be that the vesicles may contain some organic component that acts as a template for mineral nucleation. These vesicles or secretion granules may be transported to the mineralization front where crystal growth would occur (Linde 1981).

The results of the present study support the role of the Mg^{2+}-,Ca^{2+}-ATPase as outlined by Granström et al. The

localization of the enzyme on the components of the secretory pathway, especially the granular ER, would indicate that Ca^{2+} ions were being taken up from the cell, possibly as a form of homeostasis. The synthesis of proteins in the granular ER may require some of the Ca^{2+} ions that were taken up from the cell cytoplasm. In the Golgi apparatus, this calcium may be concentrated, and the proteins, possibly collagen precursors, may be seeded with calcium. The Golgi apparatus may or may not take in more calcium, depending upon the existence of the ATPase on its membranes, which was not conclusively determined in this study. The secretion granules, having the enzyme bound to their membranes, may be transported through the cell and cell processes, constantly accumulating more Ca^{2+} ions and increasing the calcium concentration associated with the protein component. When transported out of the cell, this component may act as the template for the mineral nucleating mechanism. Calcification foci, like those seen in Figure 38, would be created and mineralization of the osteodentin would proceed. The intracellular vesicles may accumulate Ca^{2+} ions from the cells via the enzymatic "calcium pump", and transport the calcium out of the cell to aid in calcium homeostasis. Both of these procedures would result in the membranes of the vesicles and secretion granules being absorbed by the cell membranes before being resorbed by coated vesicles of the cell. Thus the Mg^{2+}-,Ca^{2+}-ATPase

would be associated with the membranes and the coated vesicles, as seen in Figures 39, 42, and 45, although it may no longer have an active function.

The above sequence is supported by the work of Höhling and Fromme (1984). Using radioautography, they localized $^{45}Ca^{2+}$ within the granular ER of odontoblasts 10 minutes following injection. The labelled ions were followed as they moved through the Golgi apparatus and odotoblastic processes, and were found exclusively in the mineralized dentin 2 hours after injection. Other studies (Nagai and Frank 1974; Reith 1976) also support this proposed sequence. In fact, Reith (1976) demonstrated bound calcium in secretion granules of the Golgi region and the distal part of the odontoblast, corresponding to the localization of the enzyme to secretion granules in the same sites in the odontoblast-like cells.

The existence of both APase and Mg^{2+}-,Ca^{2+}-ATPase on the odontoblast-like cells of the osteodentin matrix has been established in the present study. Alkaline phosphatase is often used as a marker for osteoinductive cells (Wlodarski and Reddi 1986), and it is also associated with cells involved in the mineralization of dentin (Linde 1981, 1982, 1985a). Therefore, the odontoblast-like cells not only synthesize components of the osteodentin matrix (Karim and Pylypas 1986), but they also appear to be involved in the mineralization of this matrix. The odontoblast-like cells and osteodentin matrix may also be used as a model for the study of the many aspects of hard tissue formation, especially those of dentinogenesis.

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APPENDIX

Solutions

Perfusion solution

2% formaldehyde in 0.15 M cacodylate buffer, pH 7.2 containing 1.5 mM ATP (sodium salt)

Incubation solutions

Granström et al (1978): 50 mM Tris-Maleate buffer, pH 9.3
+
100 mM Na^+ (chloride salt)
+
50 mM K^+ (chloride salt)
$2+$
3 mM Ca^{2+} (chloride salt)
5 mM ATP (sodium salt)
5 mM Cadmium acetate

The final pH of the solution was adjusted to 9.3.

Takano et al (1986): 80 mM Tris-Maleate buffer, pH 8.2
$2+$
3 mM Ca^{2+} (chloride salt)
1 mM ATP (sodium salt)
3 mM lead citrate
8% glucose

The final pH of the solution was adjusted to 9.4, so as to avoid precipitation.

Disodium Ethylenediaminetetraacetate (EDTA)

41.3 g EDTA
4.4 g NaOH pellets
0.25 M (85.6 g) sucrose

The above chemicals were placed in a volumetric flask with distilled water and heated until the solutes dissolved. The solution was cooled to room temperature and water added to make 1000 ml.

Osmium Tetroxide

2-1 g Osmium tetroxide ampoules were mixed with 100 ml of distilled water to make a 2% osmium tetroxide aqueous stock solution.

Epon Mixture

Stock solution A

Epon resin 812.....64 ml
DDSA.....100 ml

Stock solution B

Epon resin 812.....100 ml
NMA.....89 ml

Final solution

Solution A.....5 parts
Solution B.....5 parts
DMP-30.....2%

Adriamycin (Doxorubicin Hydrochloride)

10 mg in 5 ml physiological saline (0.09%)

The concentration of the drug is 2 mg/ml, and the dose level is 5.0 mg/kg; therefore each animal received an injection of approximately 0.08 ml containing 0.15 mg of Adriamycin.

Stains

Uranyl Acetate

4 g of uranyl acetate was dissolved in 100 ml of 70% ethyl alcohol at room temperature, and stored at 4 ^{o}C.

Lead Citrate

This stain was prepared according to E. S. Reynolds (1963).

Figure 1

Diagram showing a longitudinal section of the mandibular rat incisor, schematically demonstrating the features relevant to the present study.

Apical, Middle, Incisal- areas of pulp
BP- basal pulp
SO zone- subodontoblastic cell-rich zone
Od- odontoblast layer
PD- predentin
D- dentin
Am- ameloblasts
E- enamel
C- cementum
ApF- apical foramen

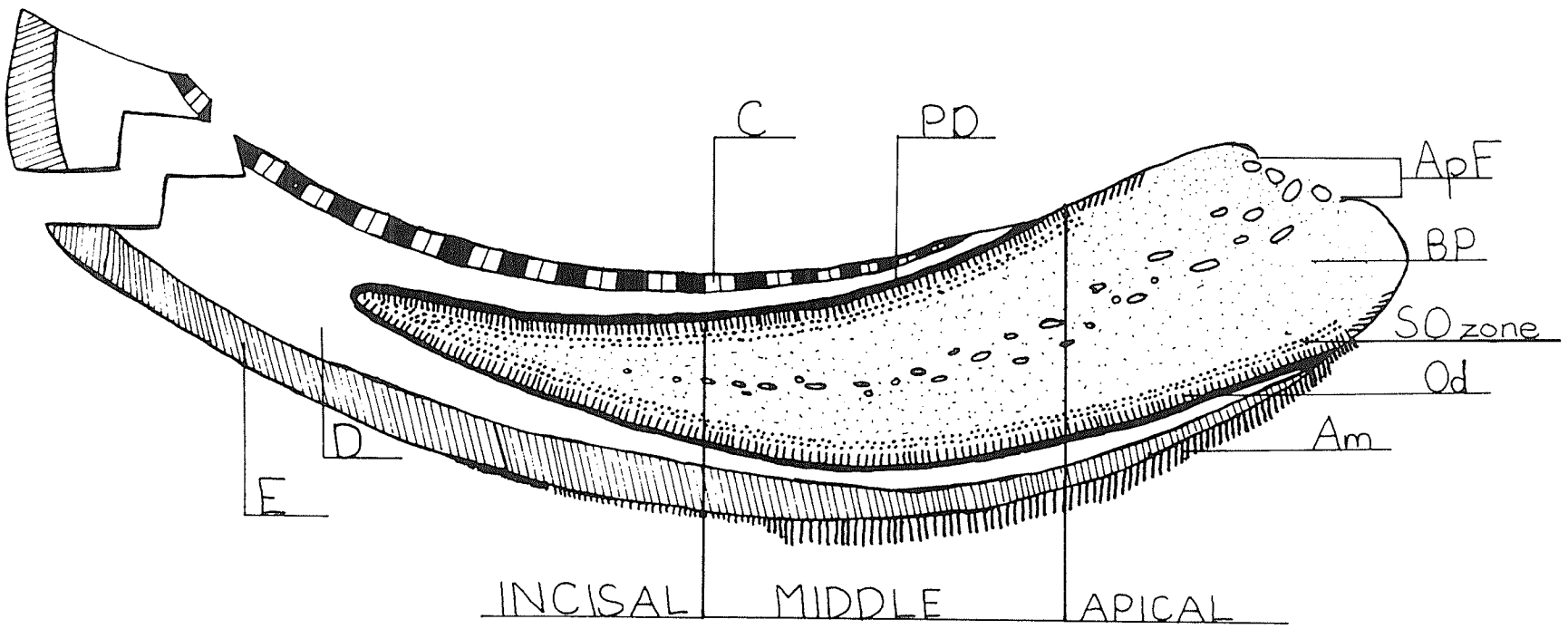
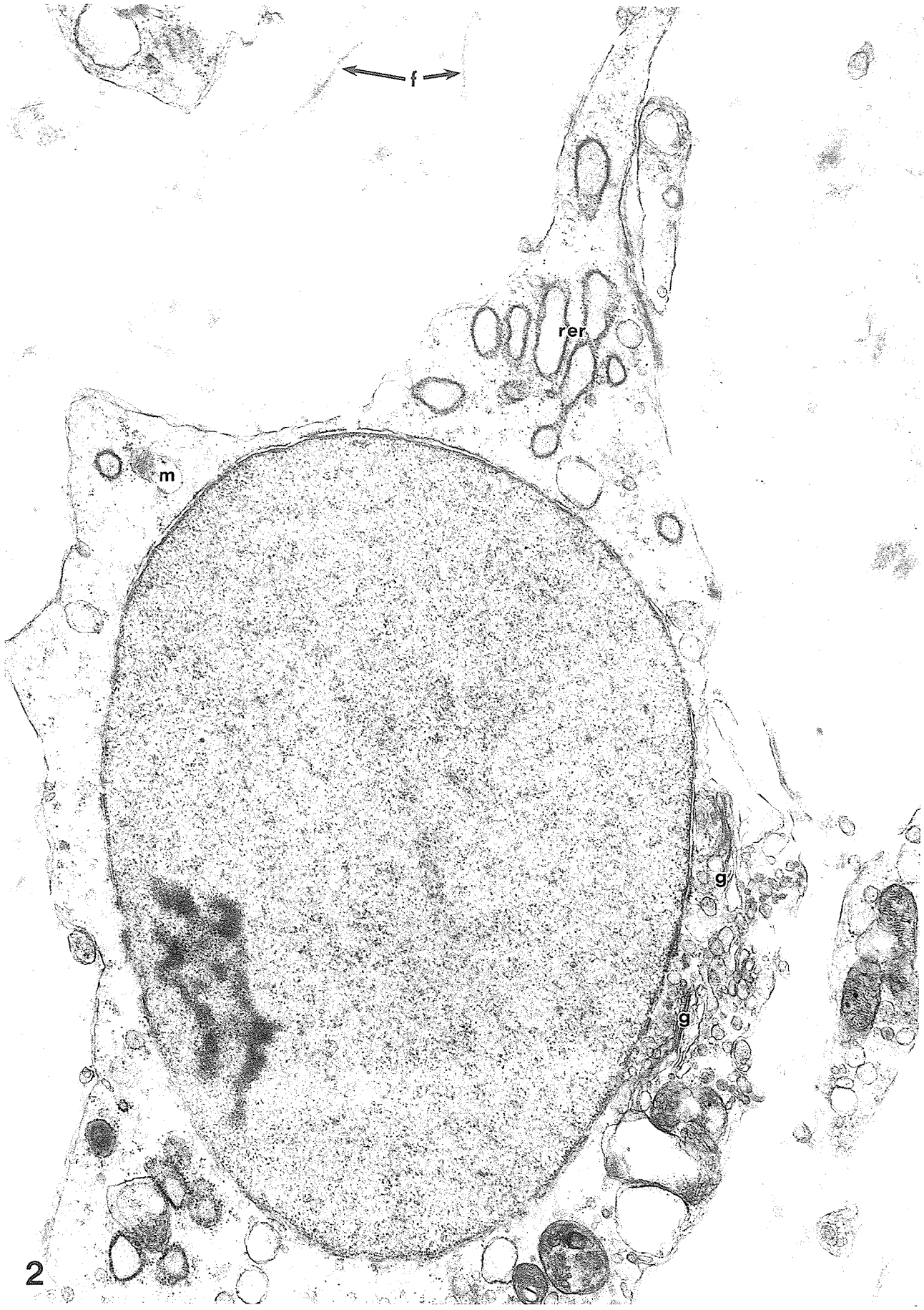

C
PD
ApF
BP
SO zone
Od
Am
D
E
INCISAL
MIDDLE
APICAL

Figure 2

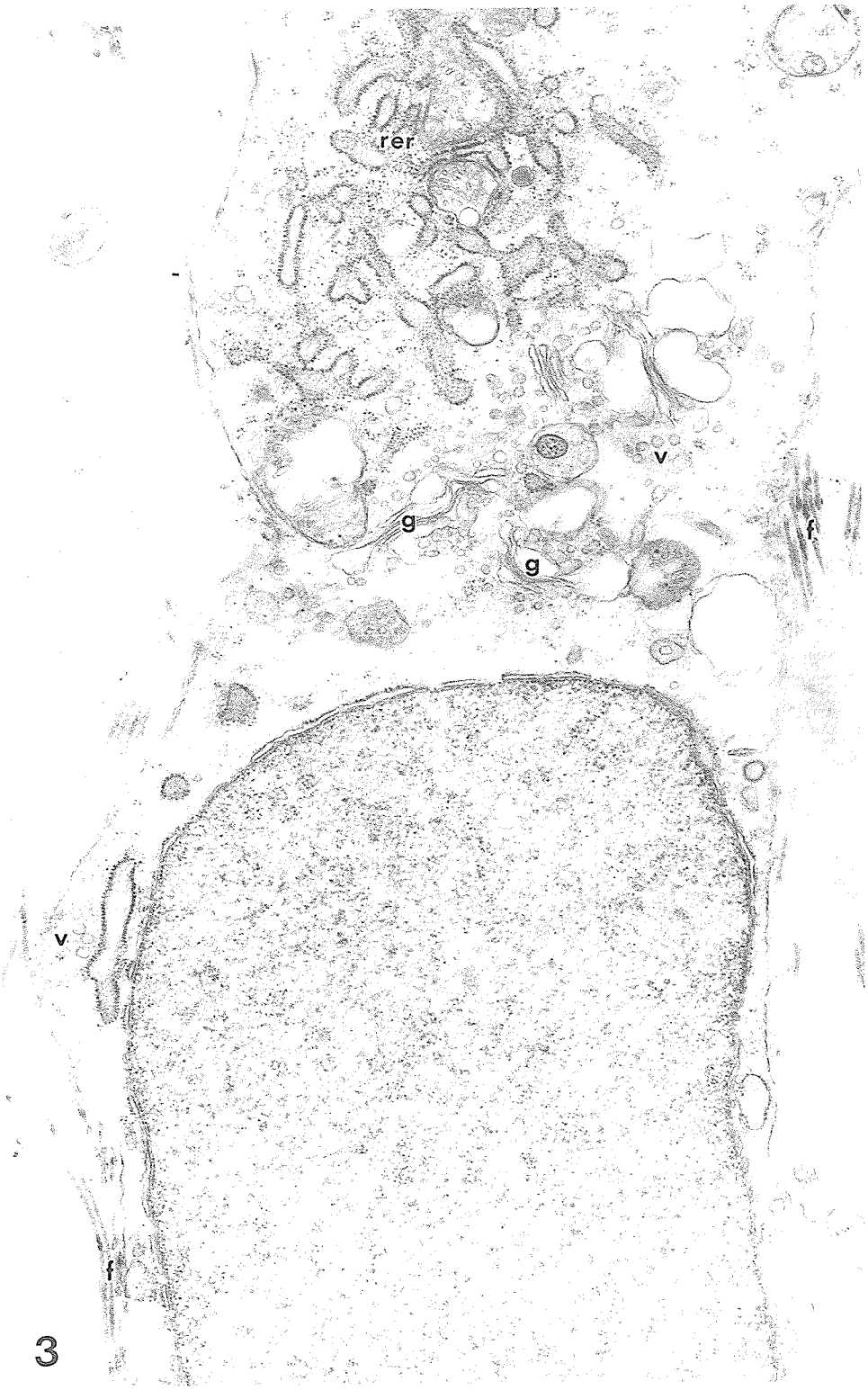
Electron micrograph of apical pulp fibroblast. Contrast stained. x19,600.
Note the lack of extensive granular ER (rer) and mitochondria (m), but the presence of a Golgi apparatus (g). The extracellular matrix is mainly amorphous with very few fibrils (f).



2

Figure 3

Electron micrograph of middle pulp fibroblast. Contrast stained. x19,600.
There is an extensive system of granular ER (rer). A Golgi apparatus (g) and vesicles (v) are present within the cell. Note the close association of the fibrils (f) of the extracellular matrix and the cellular membrane.



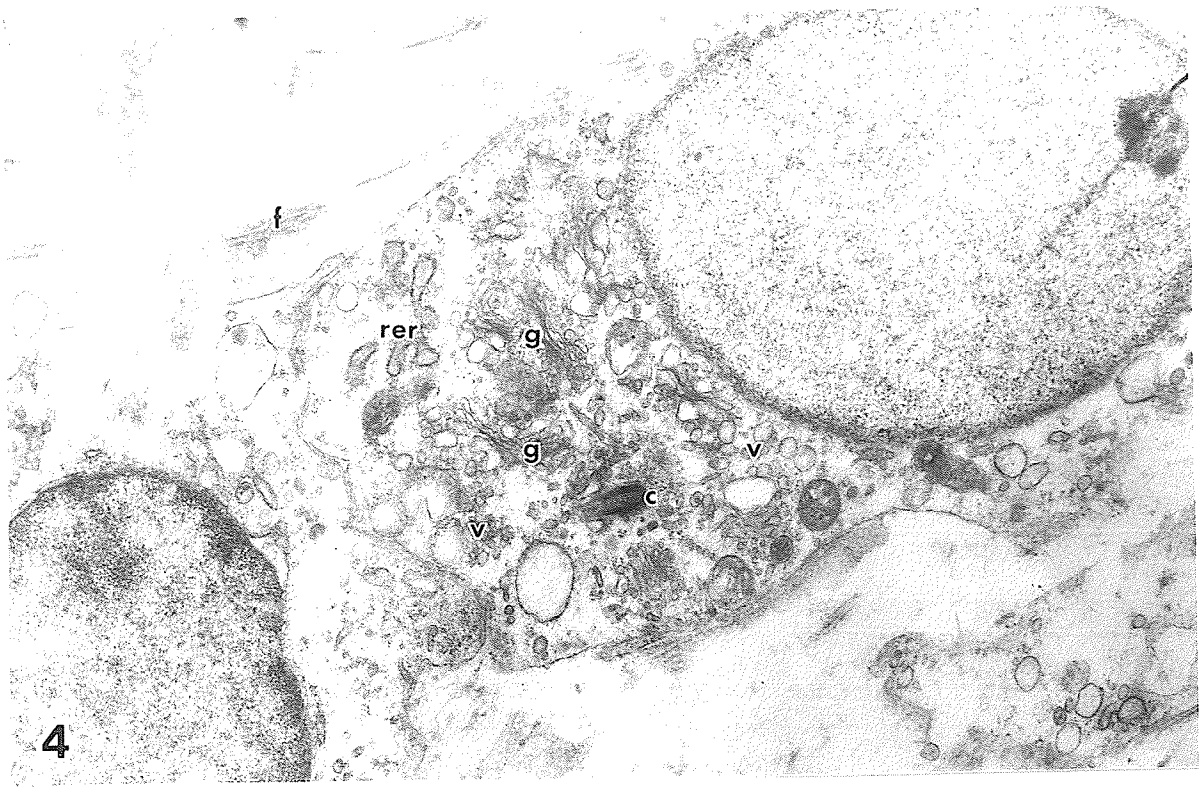
3

Figure 4

Electron micrograph of middle pulp fibroblast. Contrast stained. x13,440.
Many Golgi apparatus (g) and vesicles (v) are present in the cell. Few profiles of granular ER (rer) are present. Some fibrils (f) are present in the amorphous matrix. c-cilium.

Figure 5

Electron micrograph of incisal pulp fibroblast. Contrast stained. x19,600.
Note the fibrils (f) sequestered in the cytoplasm and associated with the membrane (arrowheads). The fibrils are also found in attenuated extensions of cells (inset). The fibrils of the matrix are more numerous than those seen in Figure 2. db-dense body; mvb-multivesicular body.


f
rer
g
g
v
c
v
4

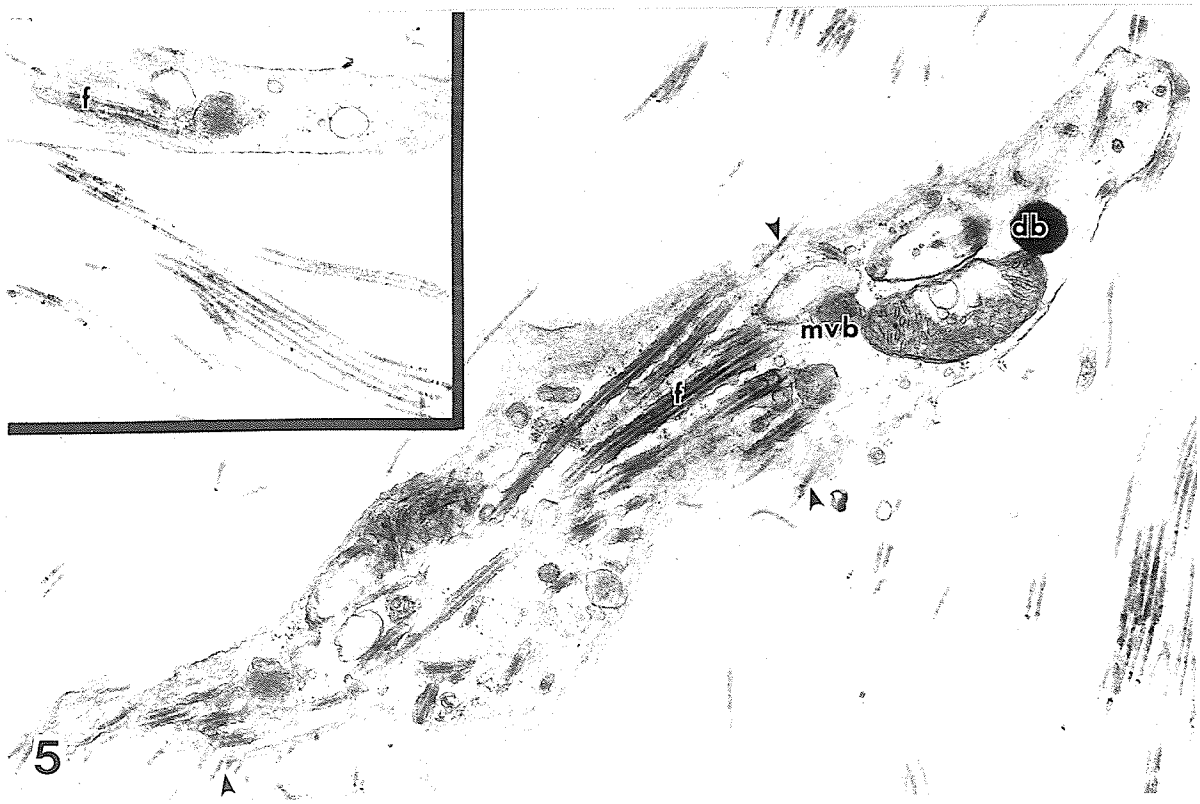
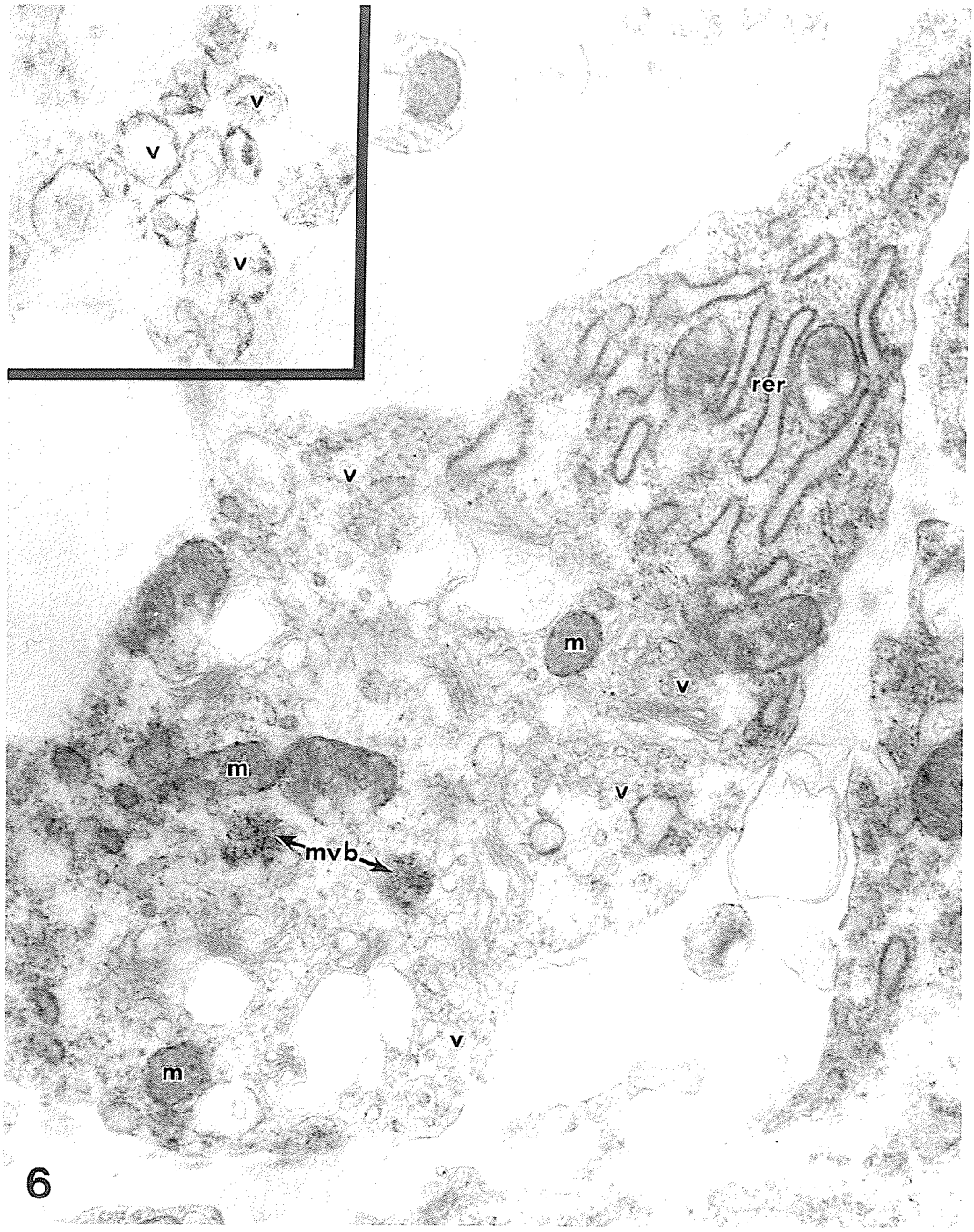

f
db
mvb
f
5

Figure 6

Electron micrograph of fibroblast from apical pulp. Incubated in medium 1. No contrast stain. x28,000. The reaction indicating alkaline phosphatase activity is seen over granular ER (rer), vesicles (v), multivesicular bodies (mvb), and mitochondria (m). The reaction on the vesicles is better seen at high magnification (x58,800) in inset.



6

Figure 7

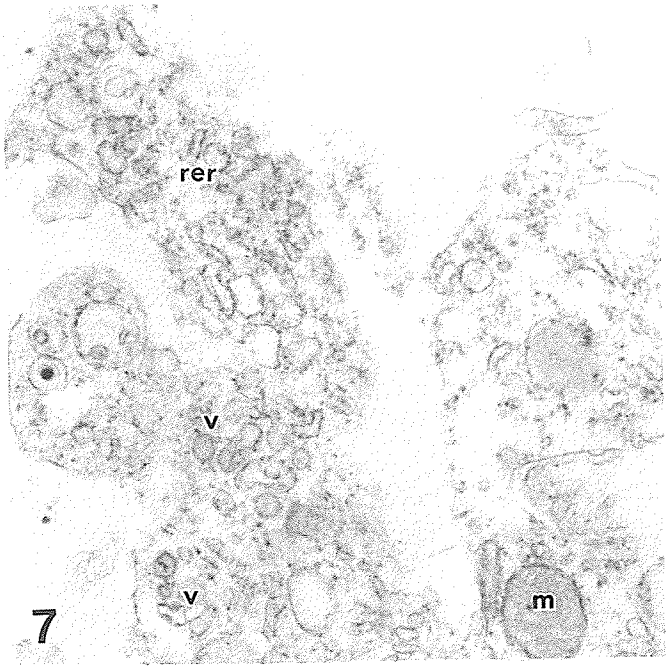
Electron micrograph of a fibroblast from apical pulp. Incubated in medium 1. No contrast stain. x 28,000. This preparation demonstrates reaction on granular ER (rer), vesicles (v), and mitochondria (m).

Figure 8

Electron micrograph of a fibroblast from the middle pulp. Incubated in medium 1. No contrast stain. x28,000. Note slight reaction on vesicles of cell (arrowheads).

Figure 9

Electron micrograph of fibroblast process from apical pulp. Incubated in medium 1. No contrast stain. x67,200. Note the reaction on vesicles (v) and along the cell membrane (arrowheads).



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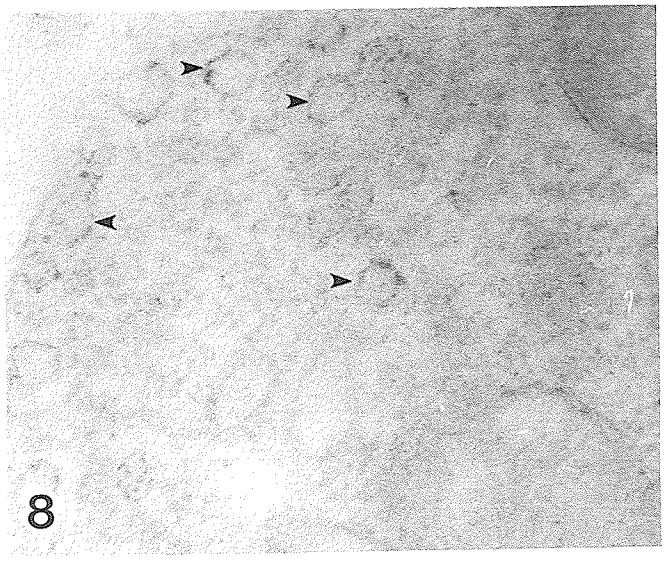

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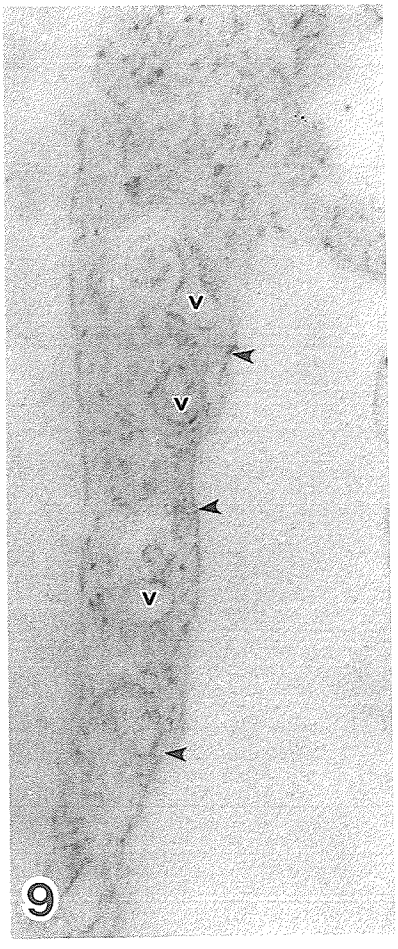


9

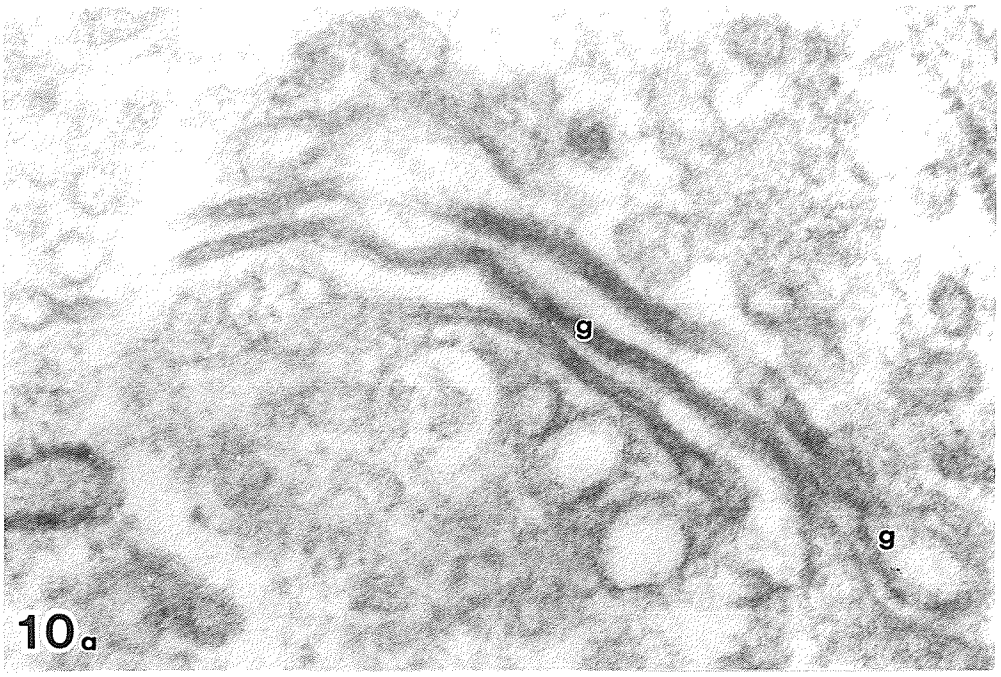
Figure 10

Electron micrographs of fibroblasts from middle pulp. Incubation in medium 1.

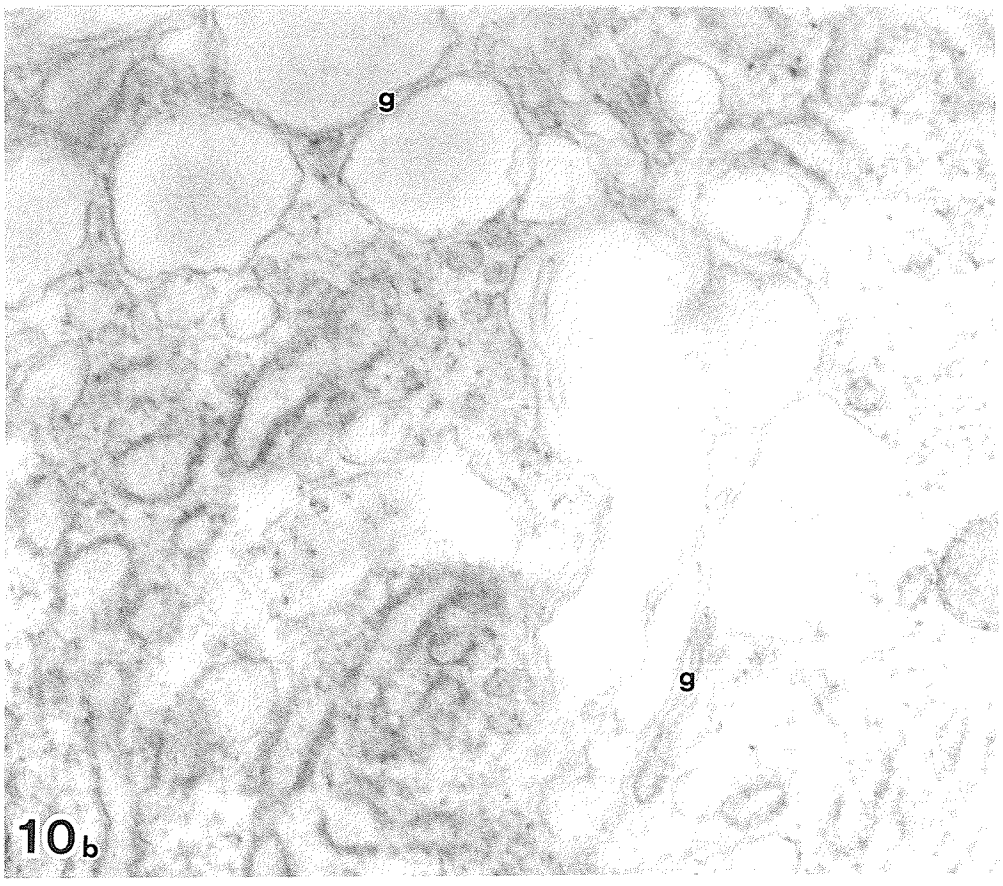
a) Contrast stained. ×67,032.

b) No contrast stain. ×58,800.

The contrast staining in a) obscures any visible reaction. Some reaction is seen on the granular ER (rer) and the mitochondria (m) but not on the Golgi apparatus (g) in b).



10a



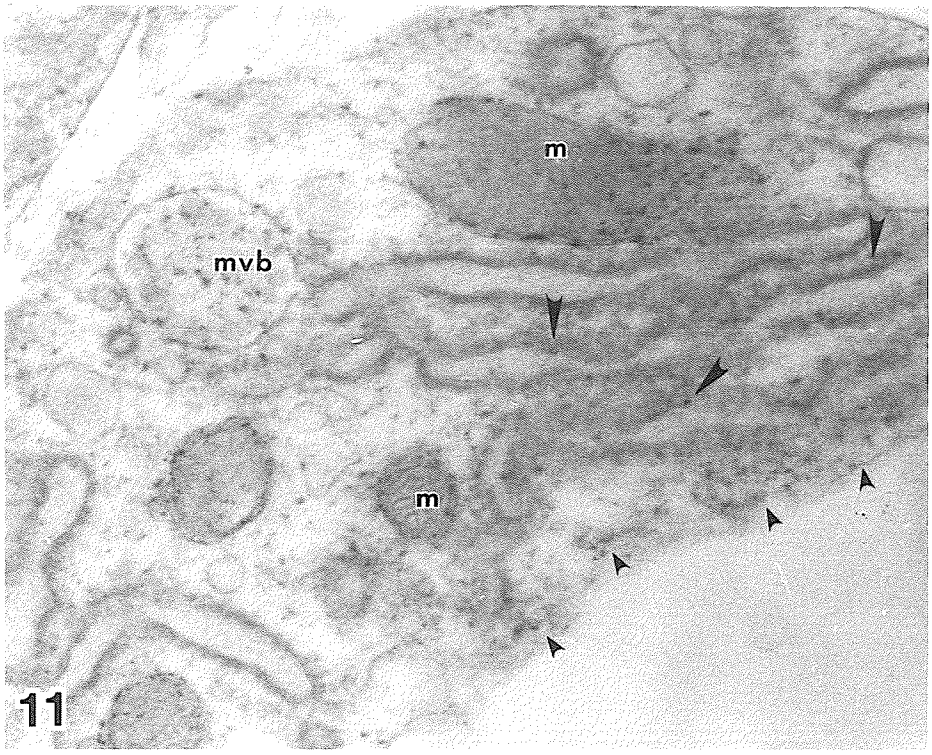
10b

Figure 11

Electron micrograph of fibroblast from middle pulp. Incubated in medium 1. No contrast stain. x50,400. The reaction is associated with the mitochondria (m), and multivesicular bodies (mvb). Slight reaction is seen on granular ER (large arrowheads) and cellular membranes (small arrowheads).

Figure 12

Electron micrograph of area from middle pulp. Incubated in medium 1. No contrast stain. x50,400. Note the sporadic reaction along the membraned structures (arrowheads), and a definite reaction on the mitochondria (m).



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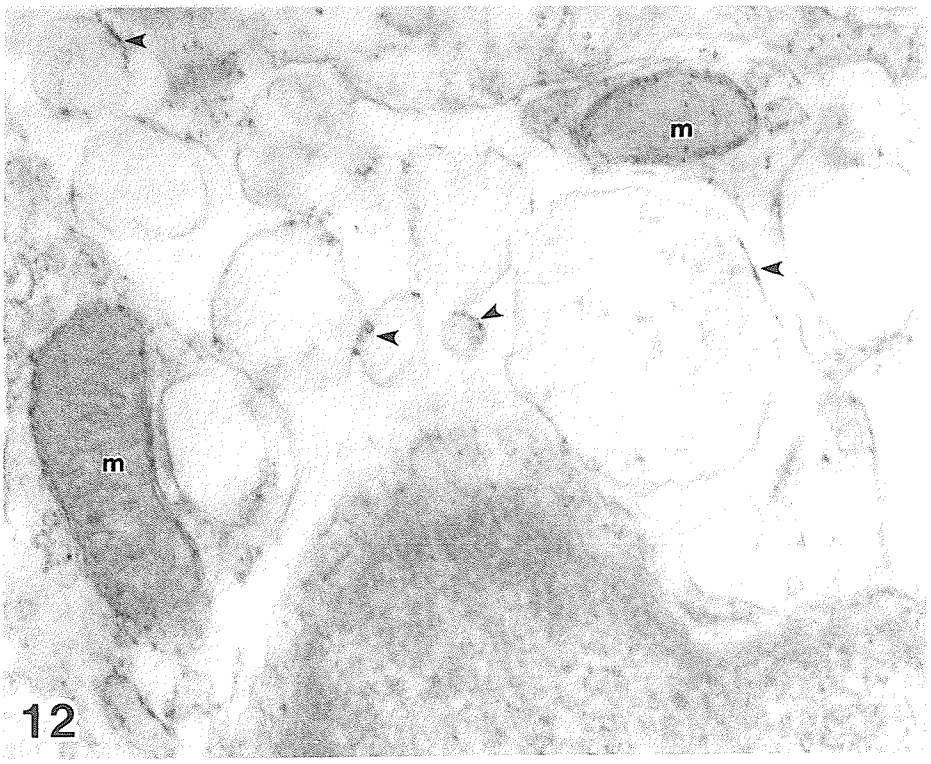
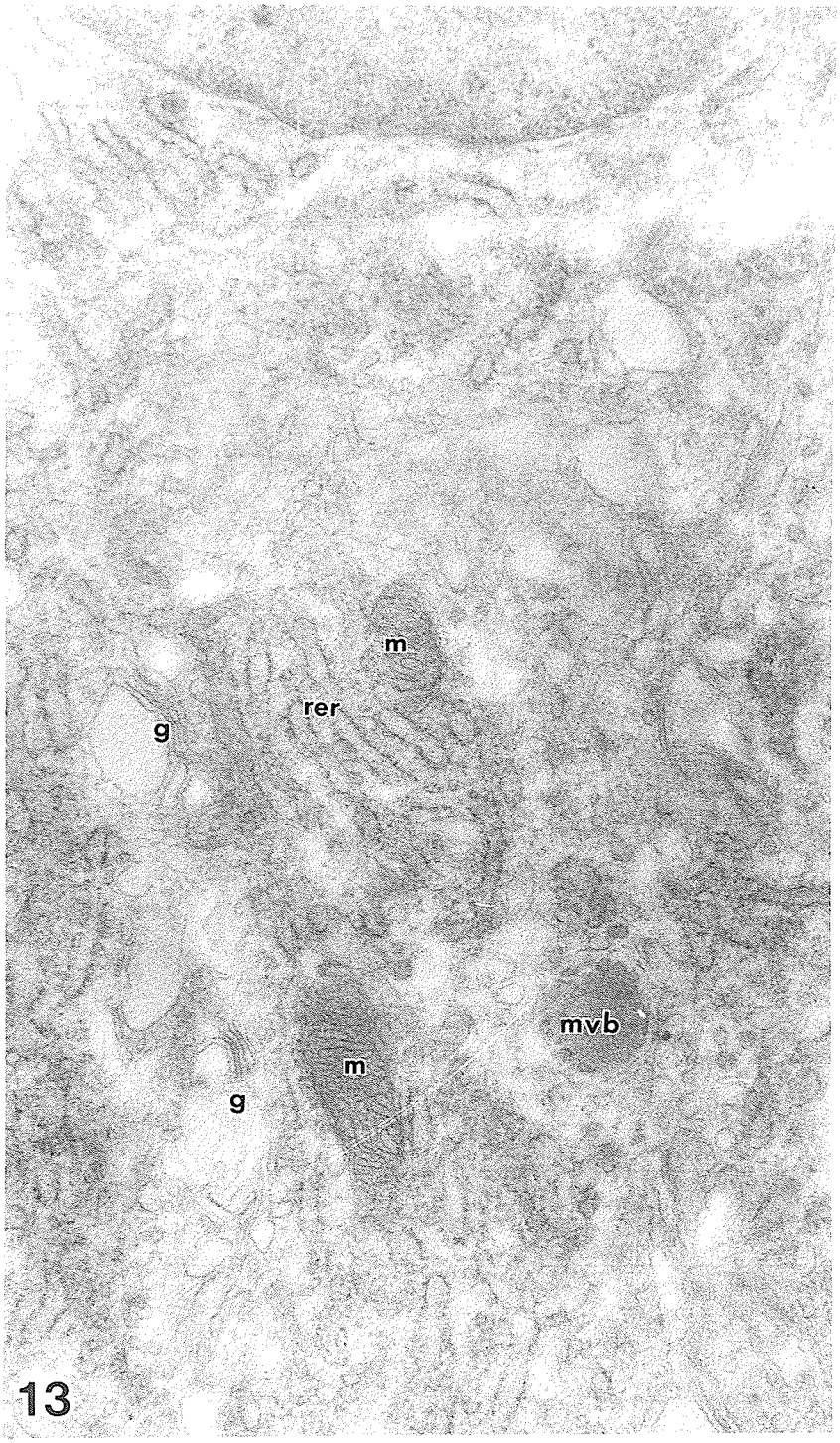


12

Figure 13

Electron micrograph of fibroblast from middle subodontoblastic zone. Heat inactivated prior to incubation in medium 2. No contrast stain. x28,00.
The reaction is diminished or completely absent from the organelles of the cell. rer-granular ER; m-mitochondria; mvb-multivesicular body; g-Golgi apparatus.



13

Figure 14

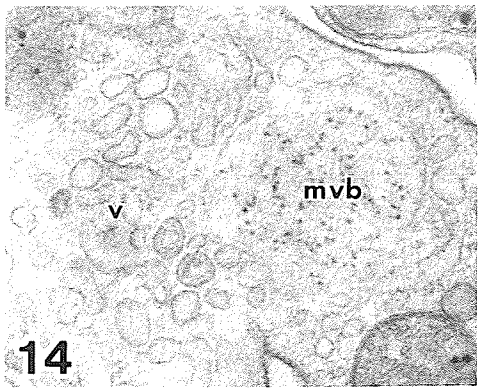
Electron micrograph of fibroblast from middle pulp.
Incubated in the presence of levamisole (medium 3).
No contrast stain. x35,112.
There is a reaction associated with the multivesicular body (mvb), but there is diminished activity on vesicles (v).

Figure 15

Electron micrograph of fibroblast from middle pulp.
Incubated in the presence of levamisole (medium 3).
No contrast stain. x19,600.
There is a reaction associated with the granular ER (rer).
db-dense body.

Figure 16

Electron micrograph of cell process from middle subodontoblastic zone. Incubated in the presence of levamisole (medium 3). Contrast stained. x67,032.
There is a reaction on the multivesicular body (MVB), the vesicles (V), and the secretion granules (sg). Note microfilaments in the cell (arrowheads).



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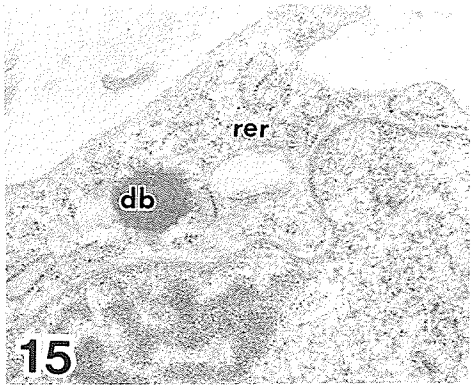

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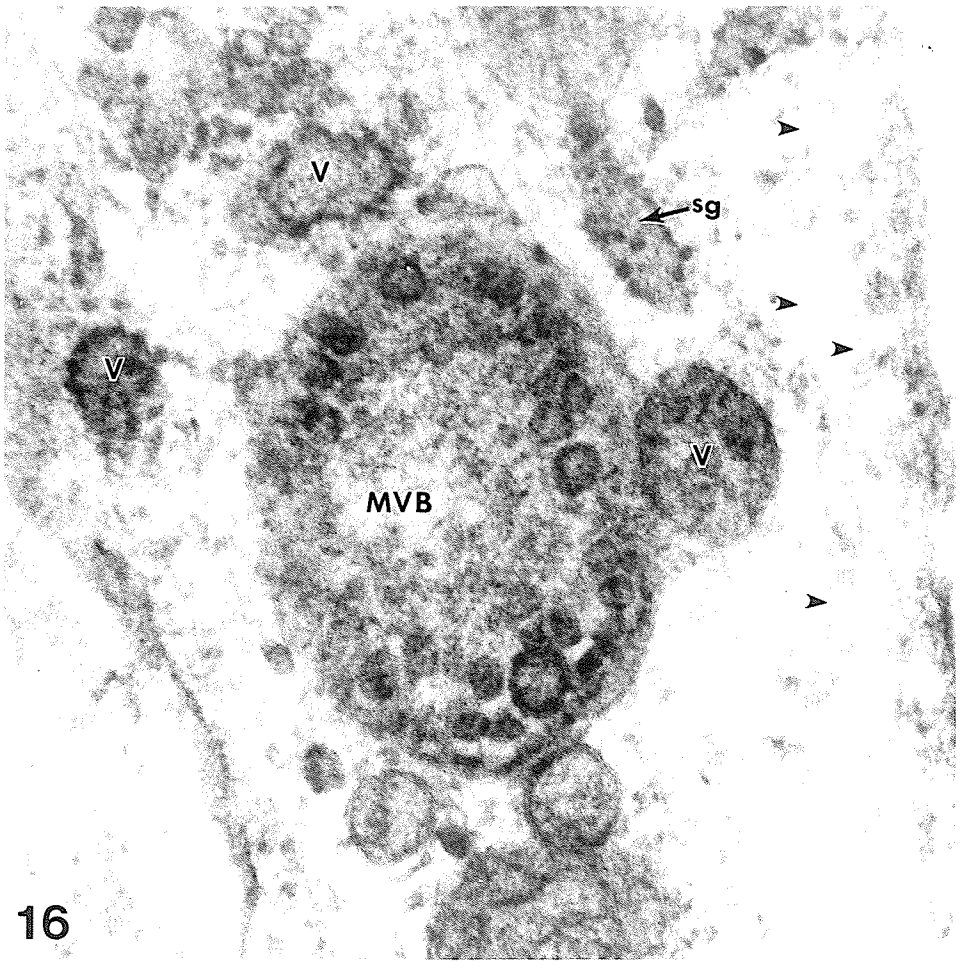


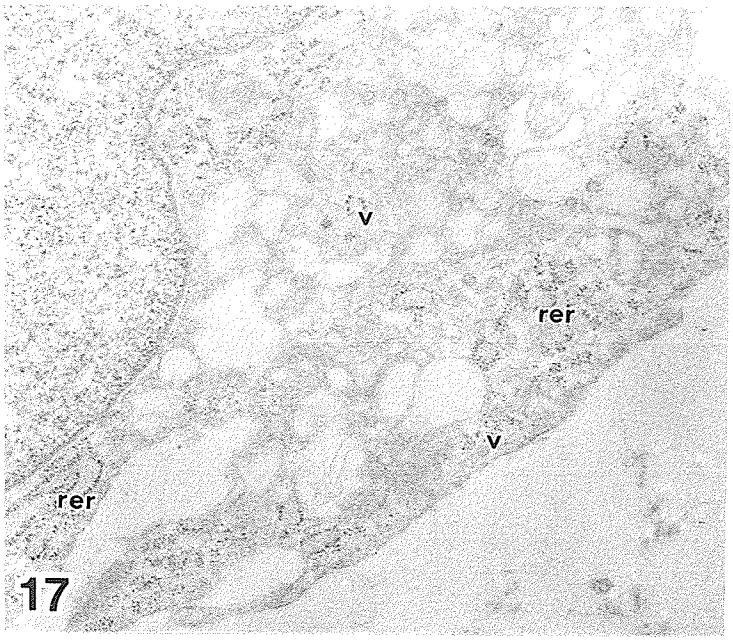
16

Figure 17

Electron micrograph of fibroblast from middle pulp.
Incubated in the presence of levamisole (medium 3).
No contrast stain. x19,600.
A reaction is seen on granular ER (rer) and vesicles (v).

Figure 18

Electron micrograph of fibroblast from middle pulp.
Incubated in the presence of Ouabain (medium 4).
No contrast stain. x42,000.
Note the reduced reaction on granular ER (arrowheads), but lack of reaction on mitochondria (m).



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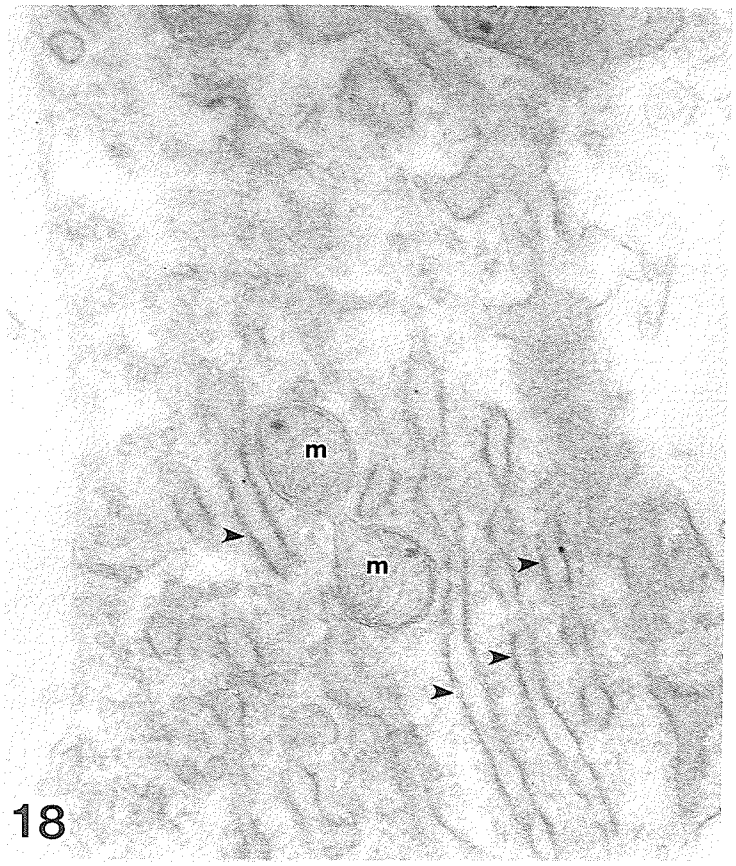


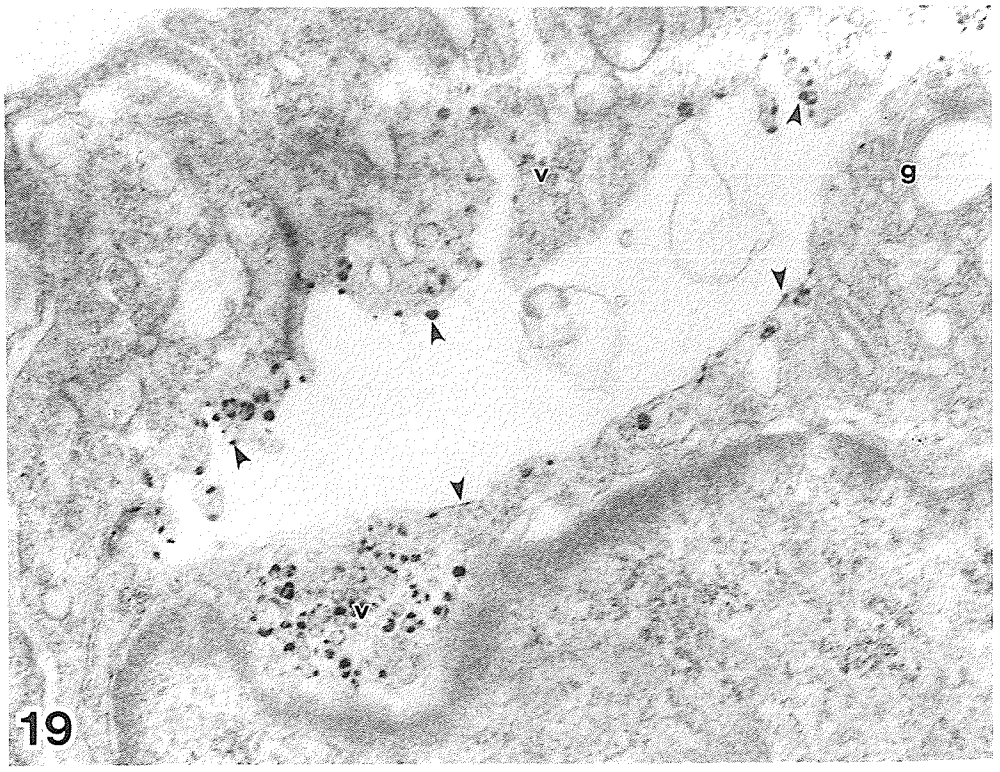
18

Figure 19

Electron micrograph of blood vessel in apical pulp. Incubated in medium 1. No contrast stain. x28,000. A reaction is seen along the luminal membrane (arrowheads) and vesicles (v) near the membrane. There is no reaction on the Golgi apparatus (g).

Figure 20

Electron micrograph of endothelial cell from apical pulp blood vessel. Incubated in medium 1. No contrast stain. x28,000.
A reaction is seen associated with vesicles (v) along the abluminal cellular membrane. L-lumen.


v
g
v
19

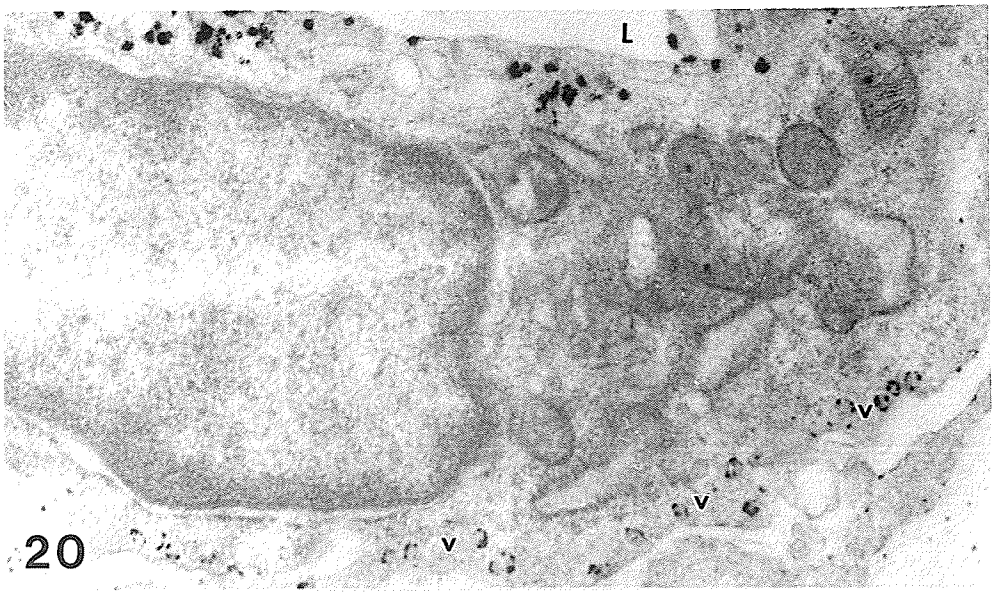
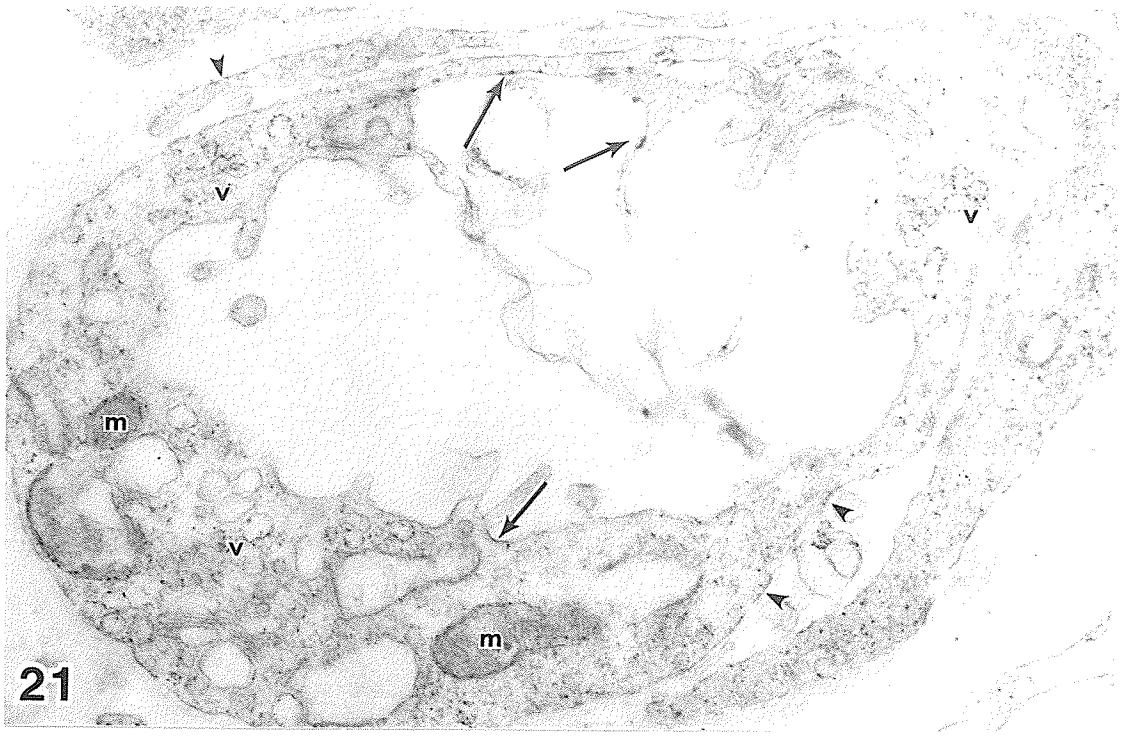

L
v
v
v
20

Figure 21

Electron micrograph of blood vessel from middle pulp. Incubated in medium 1. No contrast stain. x28,000.
A reaction can be seen on the luminal (arrows) and abluminal (arrowheads) membranes. There is also a reaction on vesicles (v) and mitochondria (m).

Figure 22

Electron micrograph of an endothelial cell from an apical blood vessel. Incubated in medium 1. No contrast stain. x28,000.
A reaction is seen on vesicles (v) and on the abluminal membrane (arrowheads). L-lumen.


v
v
m
v
m
21

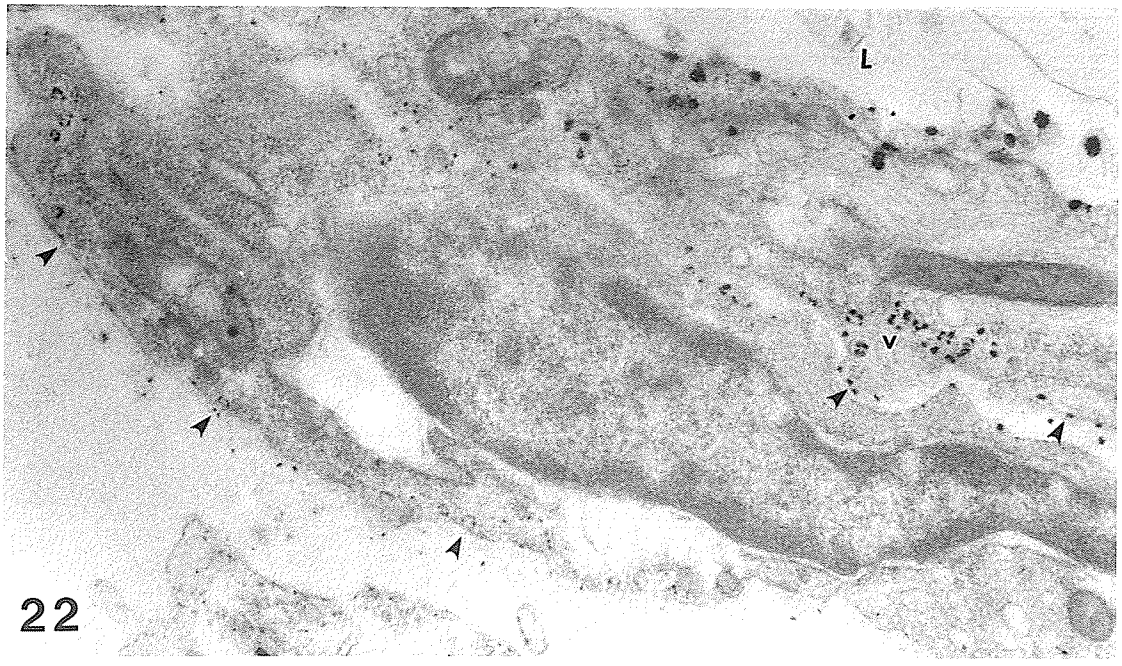

L
v
22

Figure 23

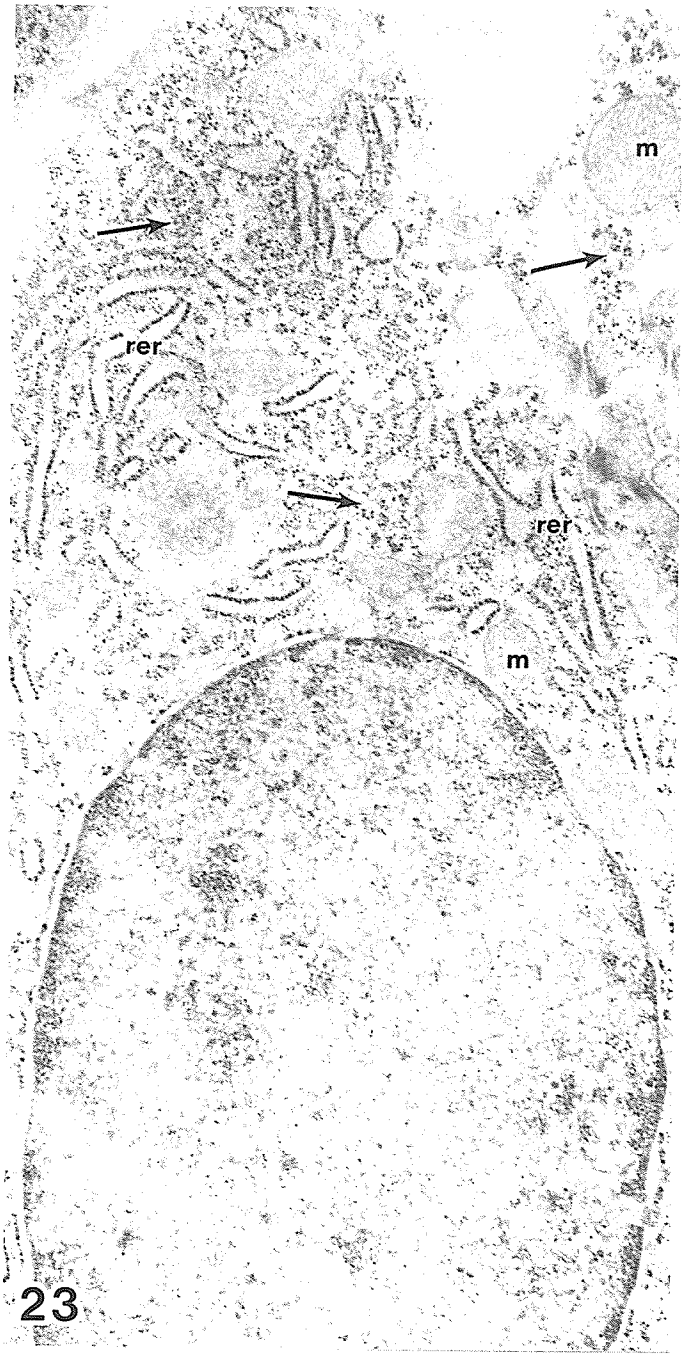
Electron micrograph of an odontoblast-like cell, 3 days following Adriamycin administration. Incubated in medium 1. No contrast stain. x25,200.
Note a reaction associated with granular ER (rer) and ribosomes (arrows). There is an absence of reaction on the mitochondria (m).

Figure 24

Electron micrographs of secretion granules from odontoblast-like cells, 3 days following Adriamycin administration. Contrast stained. x50,400.
Secretion granules can be seen in the odontoblast-like cells in a) and in the cut process of the cells in b).

Figure 25

Electron micrograph of osteodentin matrix, 3 days following Adriamycin administration. Incubated in medium 1. Contrast stained. x50,400.
A reaction is associated with cut cellular extensions in the matrix (arrowheads). Note collagenous fibres of matrix (F).



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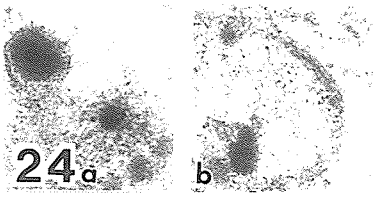
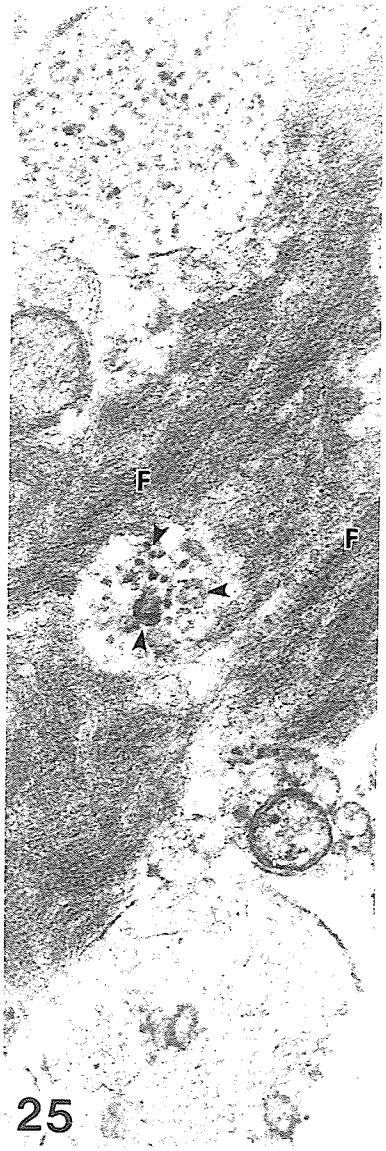




25

Figure 26

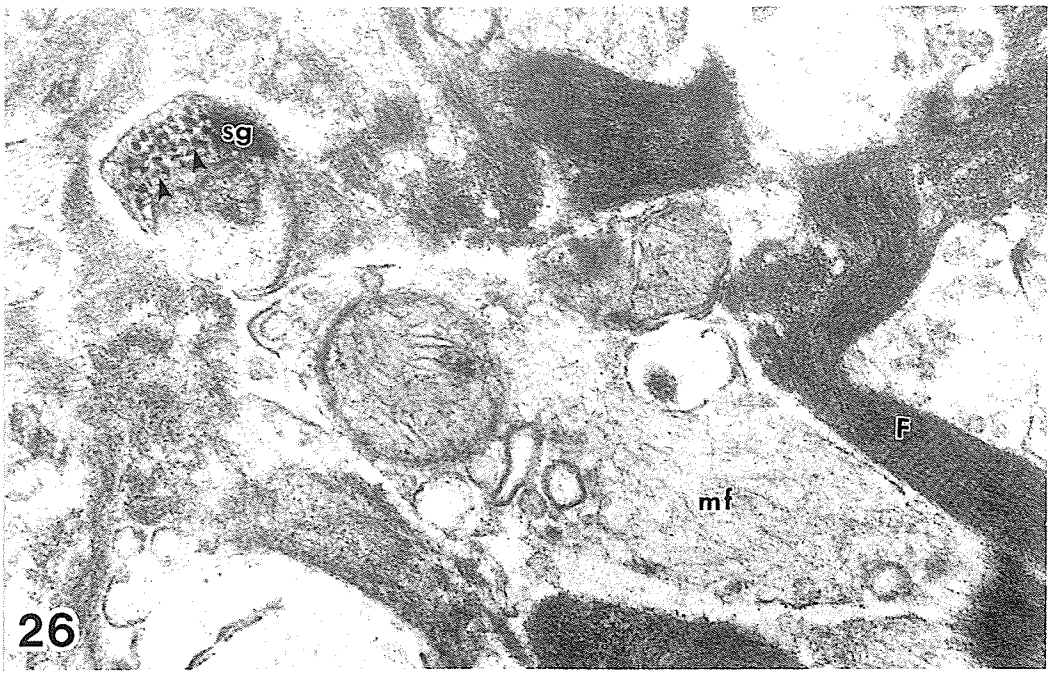
Electron micrograph showing cellular extensions within osteodentin matrix, 3 days following Adriamycin administration. Incubated in medium 1. Contrast stained. x50,400.
The reaction is associated with a secretion granule (sg) and also within the cellular process (arrowheads). Note collagenous fibres (F) within the matrix and microfilaments (mf) within a cellular process.

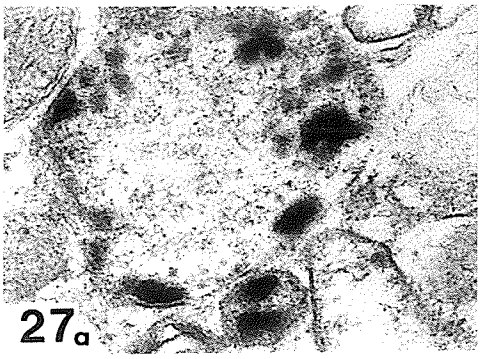
Figure 27

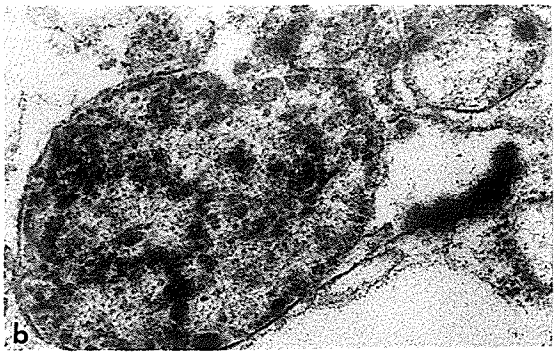
Electron micrographs of cellular processes within osteodentin matrix, 3 days following Adriamycin administration. Incubated in medium 1. Contrast stained. x50,400.
A reaction can be seen on secretion granules (a) and on multivesicular bodies (b) within the cellular processes.

Figure 28

Electron micrograph of osteodentin matrix, 3 days following Adriamycin administration. Contrast stained. x50,400.
Note the cellular processes (P) found between the collagenous fibres (F) of the matrix. Microfilaments (mf) can also be seen within a cellular process.


sg
F
mf
26


27a


b

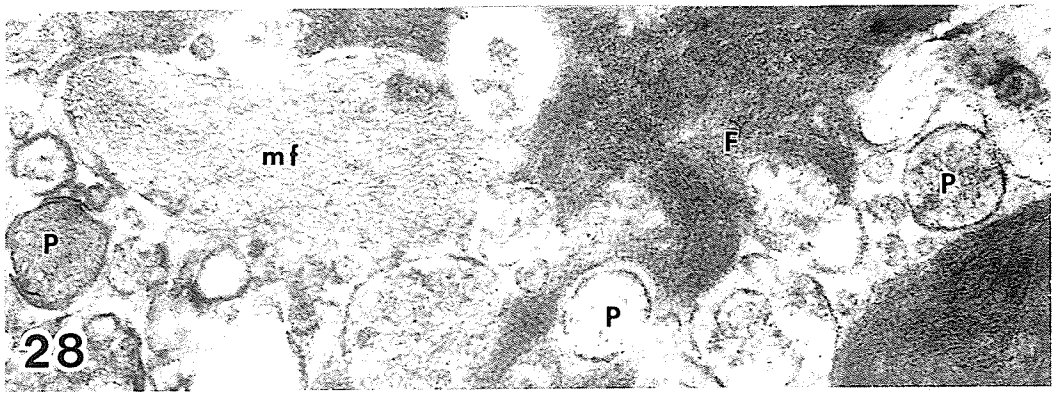
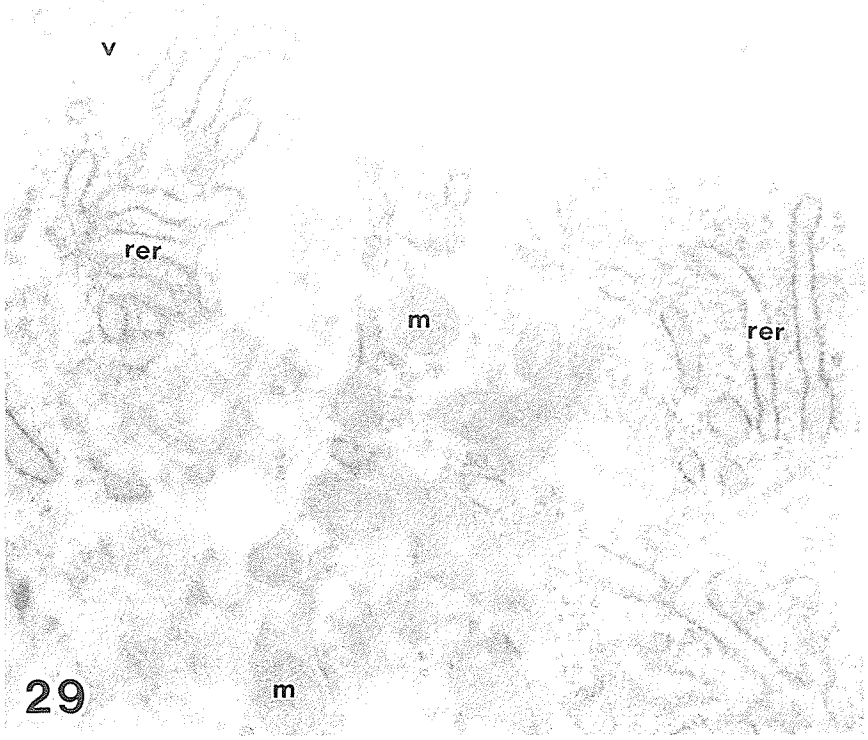

mf
F
P
P
P
28

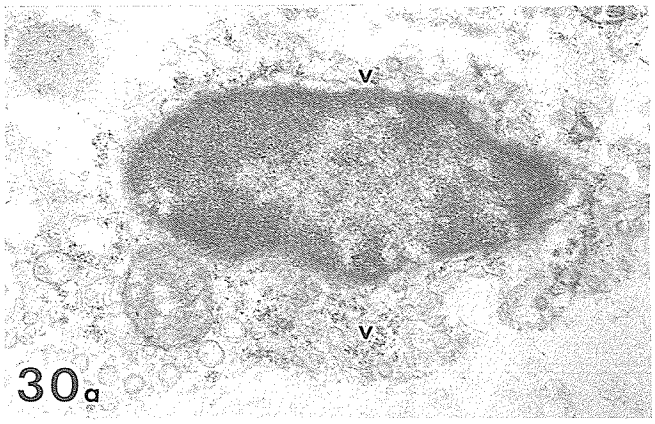
Figure 29

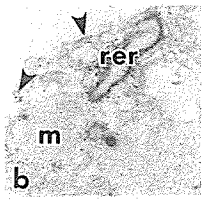
Electron micrograph of odontoblast-like cell, 3 days following Adriamycin administration. Incubated in the presence of levamisole (medium 3). No contrast stain. x25,200.
A reaction is seen on granular ER (rer), but is not present on the mitochondria (m) and vesicles (v).

Figure 30

Electron micrographs of odontoblast-like cells, 3 days following Adriamycin administration. Incubated in the presence of Ouabain (medium 4). No contrast stain. x19,600.
A reaction can be seen on vesicles (v) in a), granular ER (rer) in b) and c), and cellular membranes (arrows) in b). There is no reaction associated with mitochondria (m) in b) and c).


v
rer
m
rer
m
29


v
v
30a


rer
m
b

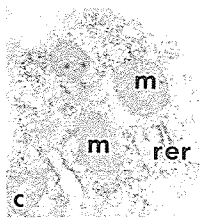

m
m
rer
c

Figure 31

Electron micrograph showing crystals from a mineralized section of osteodentin matrix, 5 days following Adriamycin administration. No contrast stain. x19,600.
Note the rectangular shape of the crystals and their irregular arrangement.

Figure 32

Electron micrograph showing a section from demineralized osteodentin matrix, 5 days following Adriamycin administration. Contrast stained. x33,600.
Note the irregular arrangement of the collagen fibres (F) and the large amount of ground substance between the fibres of the matrix.


31

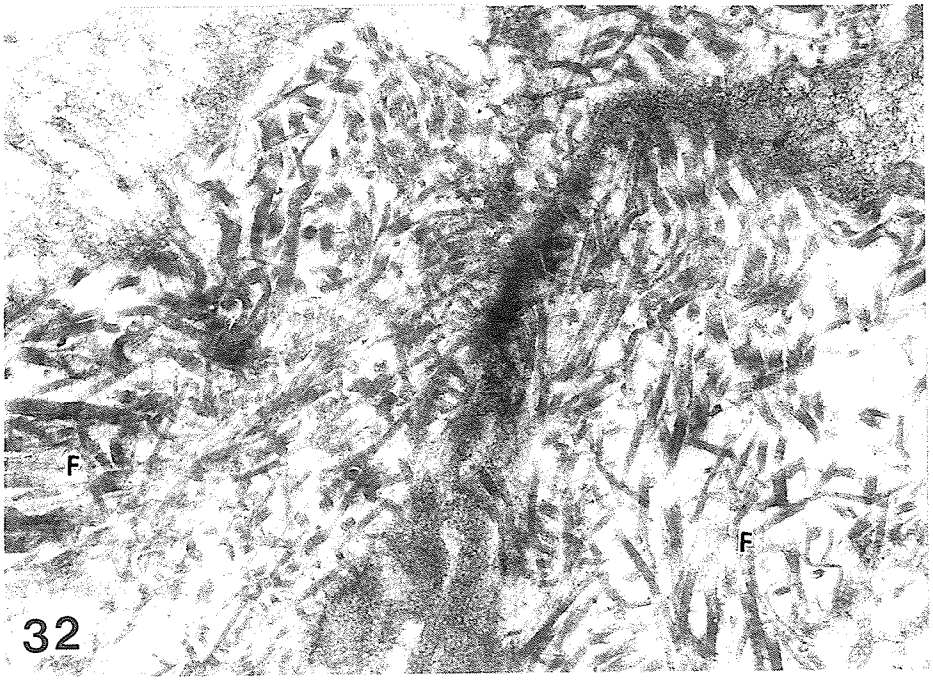
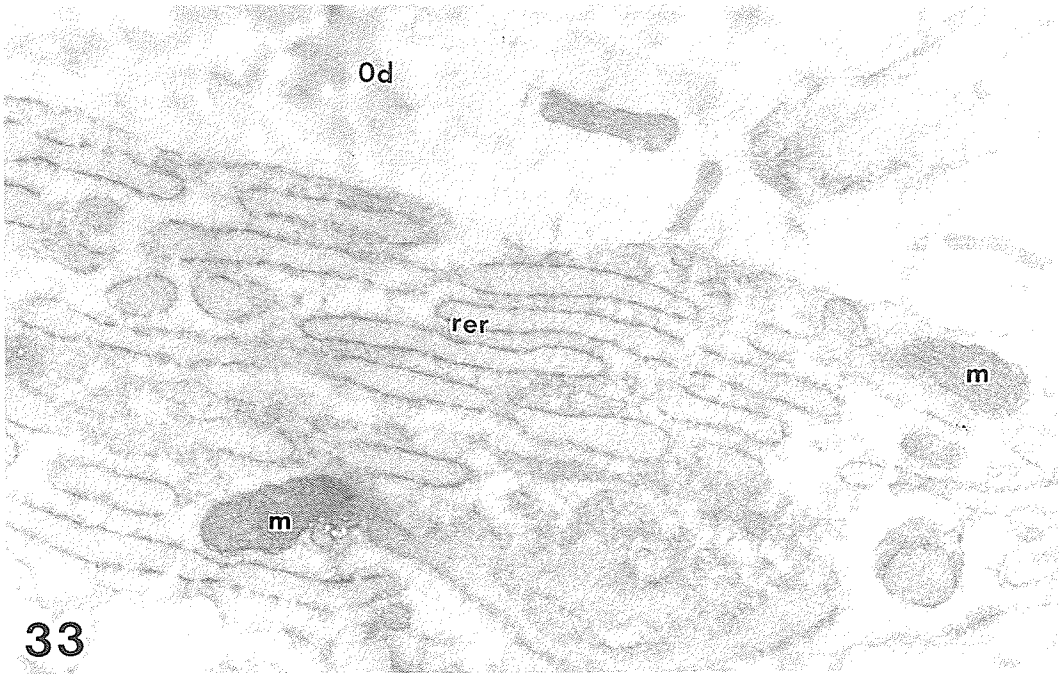

F
F
32

Figure 33

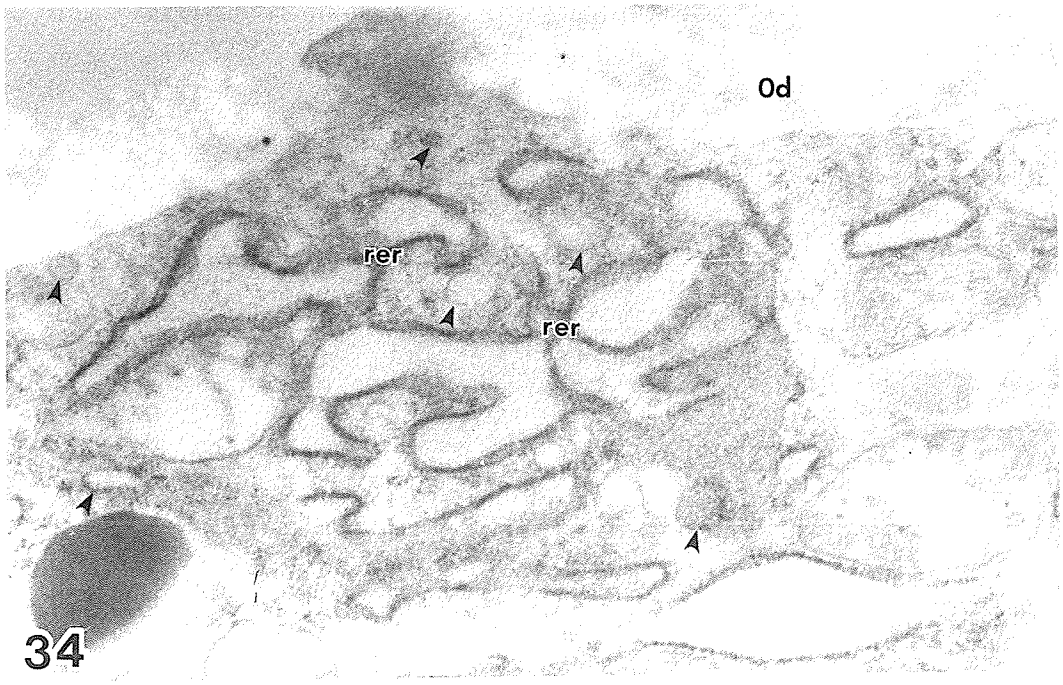
Electron micrograph of a demineralized section showing an odontoblast-like cell in osteodentin matrix (Od), 5 days following Adriamycin administration. Incubated in medium 1. No contrast stain. x25,200.
Note the diminished reaction on the granular ER (rer) and the lack of reaction on the mitochondria (m).

Figure 34

Electron micrograph of an undemineralized section showing an odontoblast-like cell, 5 days following Adriamycin administration. Incubated in the presence of levamisole (medium 3). No contrast stain. x33,600.
Note the reaction on the granular ER (rer), and the sporadic reaction on vesicles (arrowheads). Od-osteodentin.



33



34

Figure 35

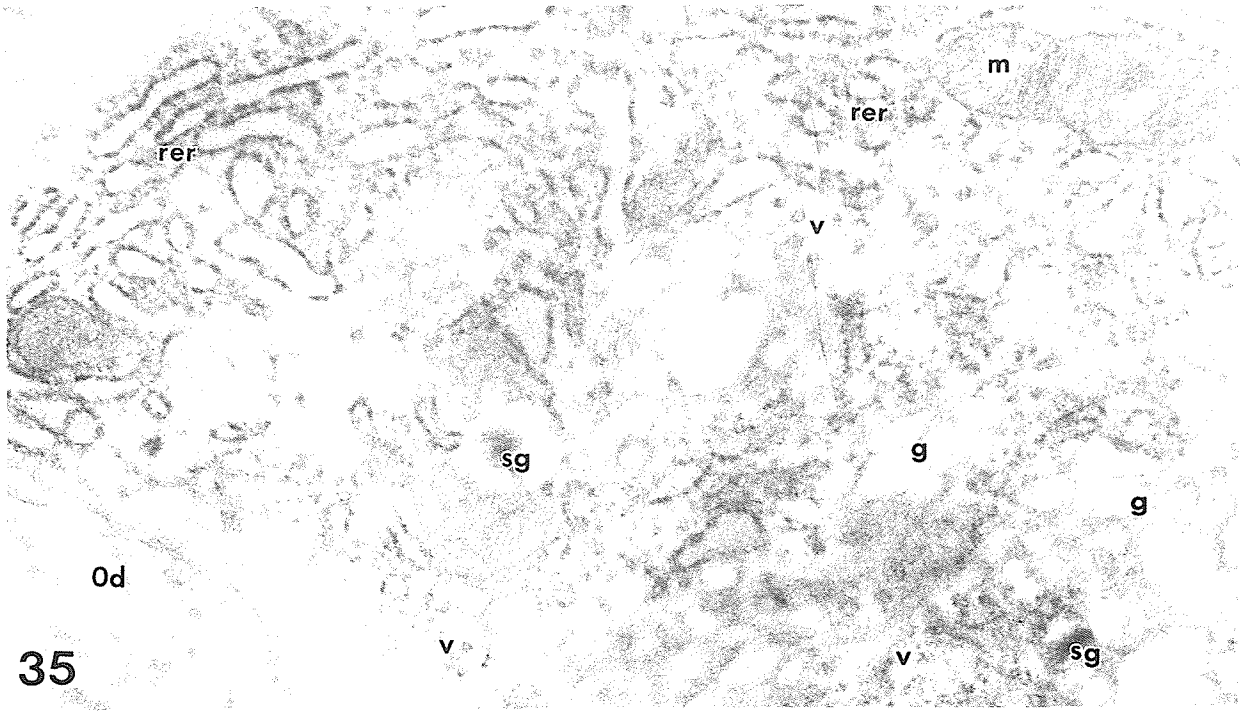
Electron micrograph of an undemineralized section showing an odontoblast-like cell, 5 days following Adriamycin administration. Incubated in the presence of levamisole (medium 3). No contrast stain. x28,000.
A reaction is seen on granular ER (rer), vesicles (v), and secretion granules (sg). No reaction is seen on mitochondria (m) or Golgi saccules (g).

Figure 36

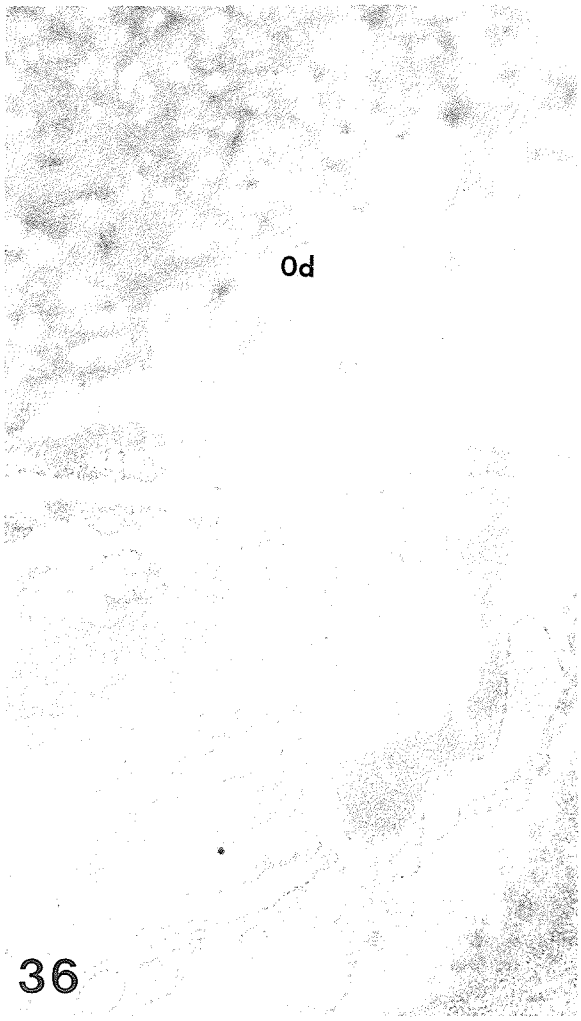
Electron micrograph of an undemineralized section of osteodentin matrix (Od), 5 days following Adriamycin administration. Heat inactivated prior to incubation in medium 2. No contrast stain. x19,600.
Note absence of reaction on organelles of odontoblast-like cell.

Figure 37

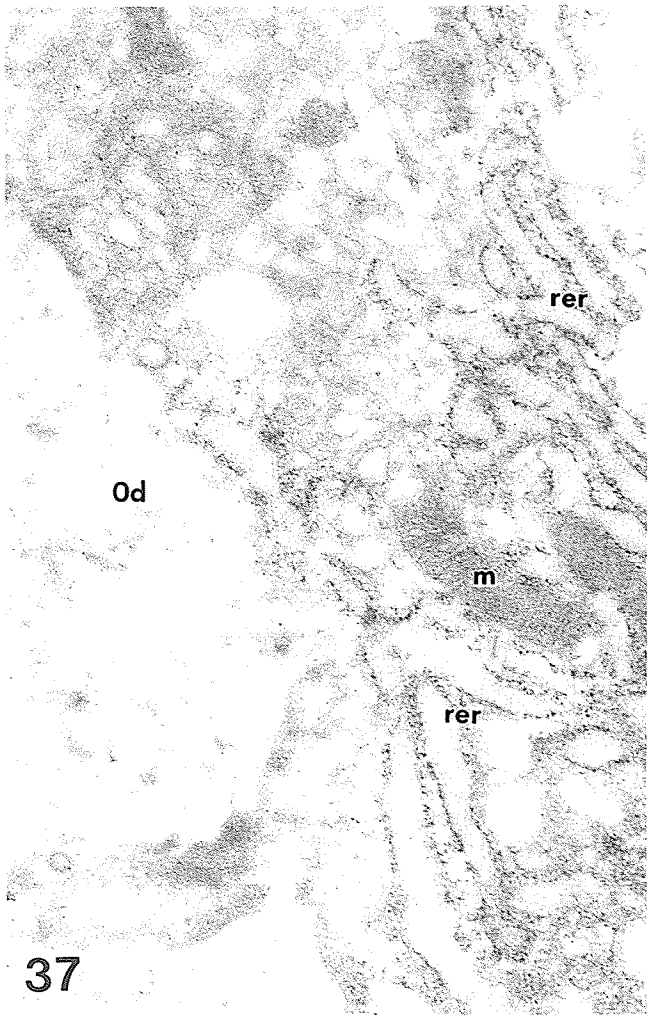
Electron micrograph of a demineralized section showing an odontoblast-like cell in osteodentin matrix (Od), 5 days following Adriamycin administration. Incubated in the presence of Ouabain (medium 4). No contrast stain. x33,600.
Note the reduced reaction on the granular ER (rer), and the absence of reaction on the mitochondria (m).



35



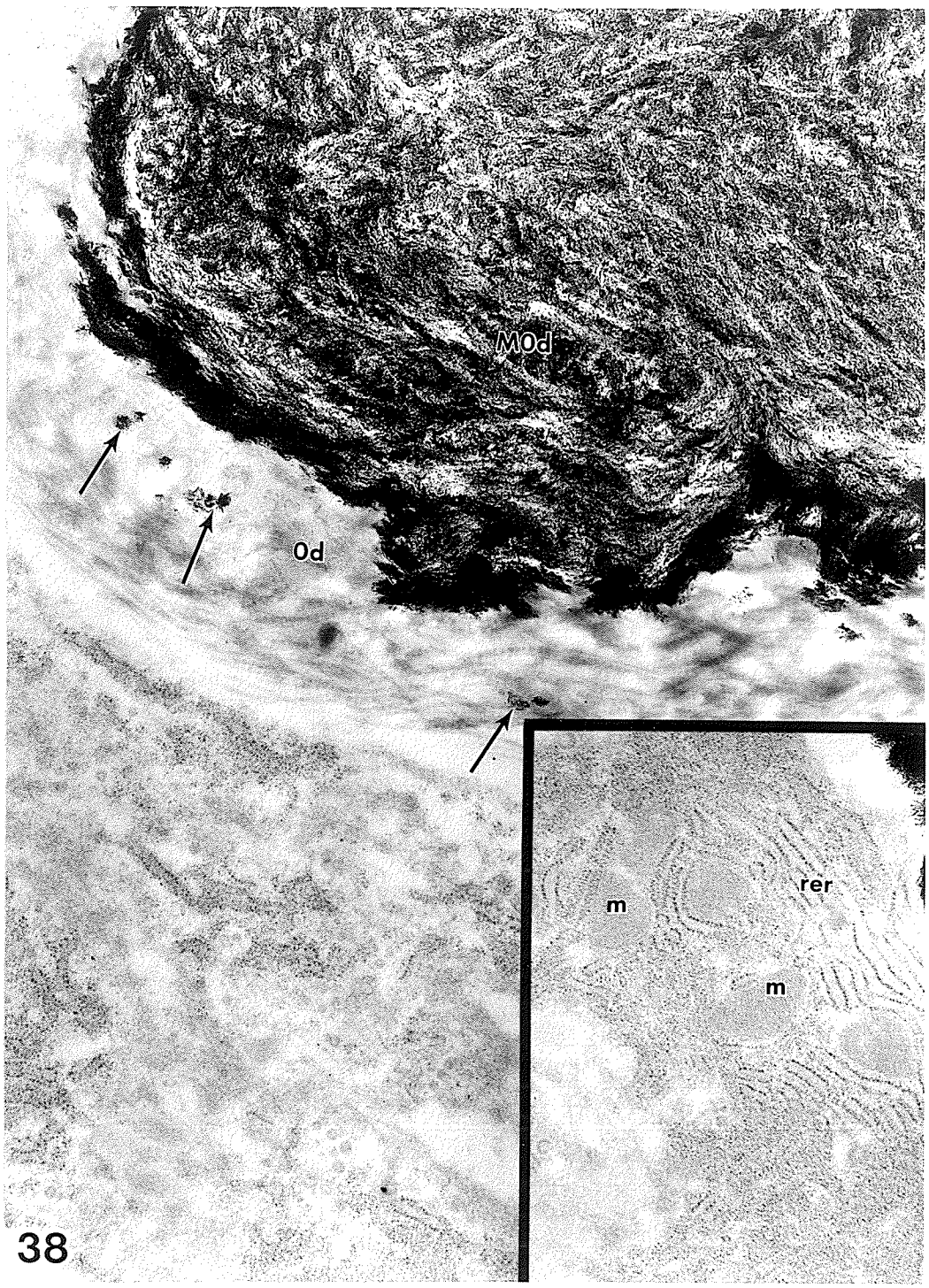
36



37

Figure 38

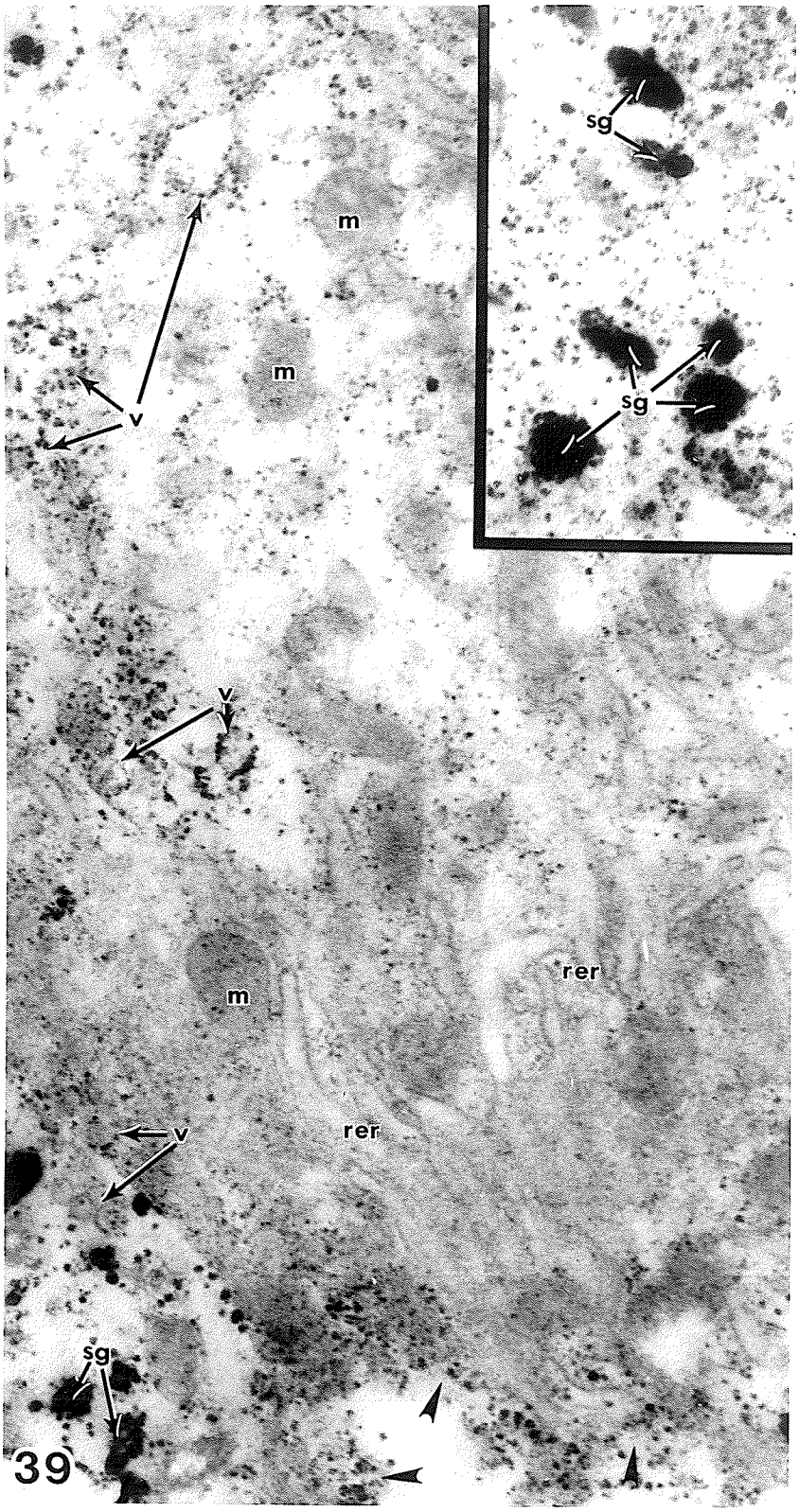
Electron micrograph of an undemineralized section showing osteodentin, 7 days following Adriamycin administration. Incubated in medium 1. No contrast stain. x19,600. Both the mineralized (MOd) and unmineralized (Od) portions of the osteodentin matrix can be seen. Mineralization foci (arrows) are associated with collagen fibres of the matrix. The odontoblast-like cells (inset) show reaction on the granular ER (rer), but not on the mitochondria (m).



38

Figure 39

Electron micrograph of an odontoblast-like cell, 7 days following Adriamycin administration. Incubated in medium 1 for 60 minutes. No contrast stain. x28,000.
There is a reaction on vesicles (v) and along the cellular membrane (arrowheads). Reaction is intense on secretion granules (sg; inset x33,600). A reaction on granular ER (rer) is not visible and is absent on mitochondria (m).


m
m
v
v
sg
sg
v
m
rer
rer
v
sg
39

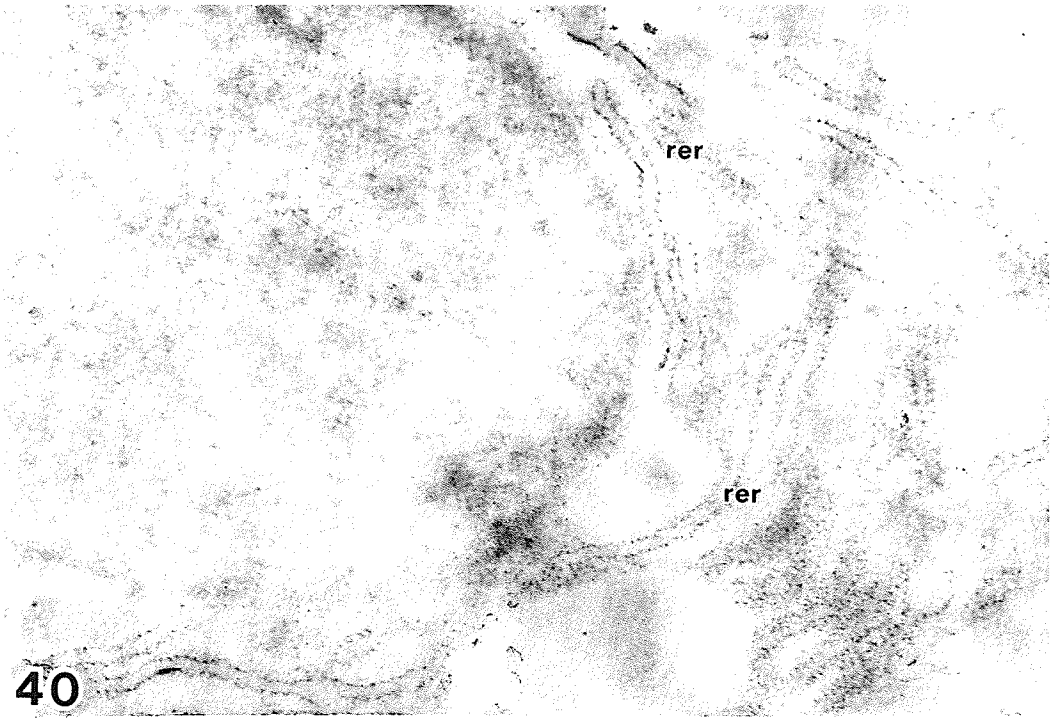
Figures 40-45 are sections from material incubated in media described by Takano et al (1986).

Figure 40

Electron micrograph of an odontoblast-like cell, 7 days following Adriamycin administration. Heat inactivated prior to incubation in medium 2. No contrast stain. x25,200. Note the reduced reaction along the granular ER (rer).

Figure 41

Electron micrograph of odontoblast-like cells and osteodentin matrix (Od), 7 days following Adriamycin administration. Incubated in the presence of levamisole (medium 3). No contrast stain. x16,800.
A reaction is associated with secretion granules (sg), and the cellular membrane (arrowheads).


rer
rer
40

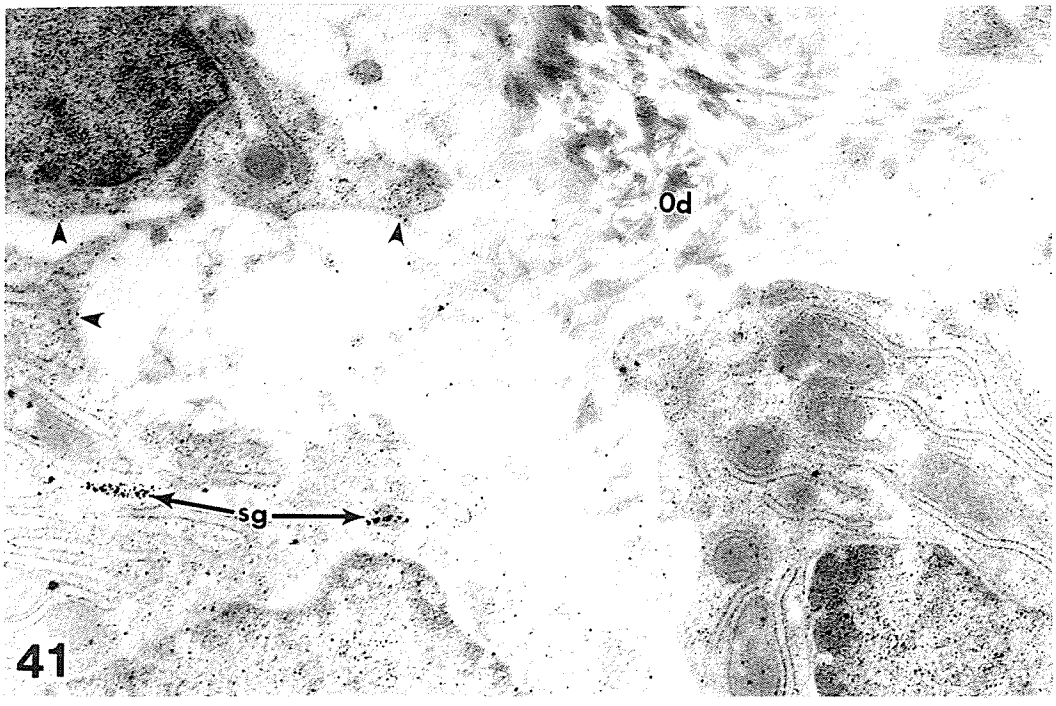
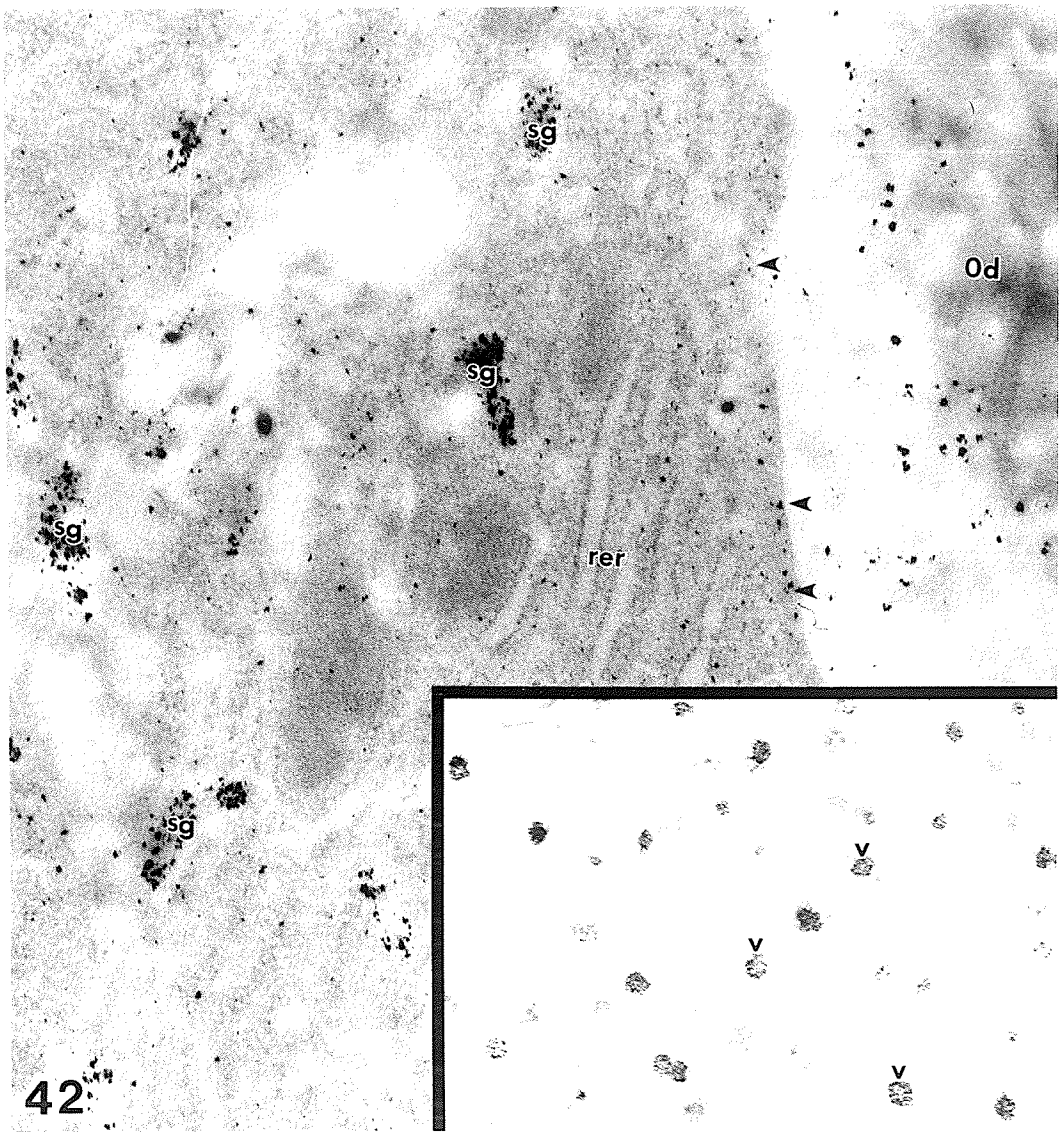

0d
sg
41

Figure 42

Electron micrograph showing an odontoblast-like cell in osteodentin matrix (Od), 7 days following Adriamycin administration. Incubated in the presence of levamisole (medium 3) for 50 minutes. No contrast stain. x33,600. A reaction is seen associated with secretion granules (sg), vesicles (v; inset x58,800), and cellular membranes (arrowheads). A reaction on the granular ER (rer) is not visible.



42

Figure 43

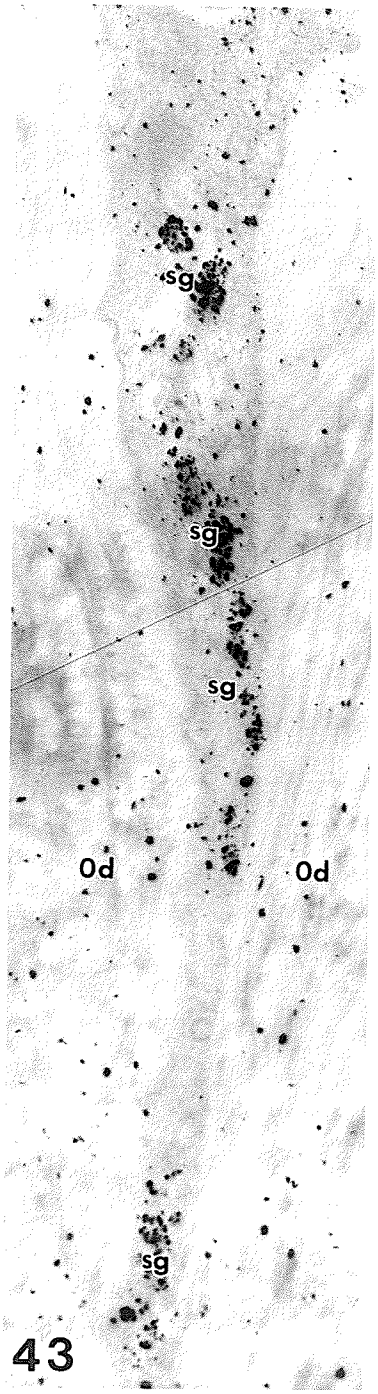
Electron micrograph of an odontoblast-like cell process within osteodentin matrix (Od), 7 days following Adriamycin administration. Incubated in the presence of levamisole (medium 3) for 50 minutes. No contrast stain. x24,000.
The reaction is seen associated with secretion granules (sg) in the process.

Figure 44

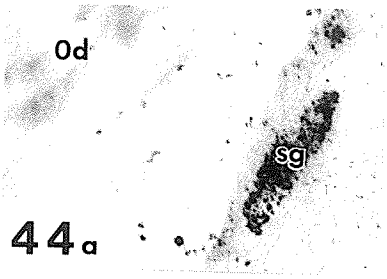
Electron micrographs of sections of osteodentin matrix (Od), 7 days following Adriamycin administration. Incubated in the presence of levamisole (medium 3) for 50 minutes. No contrast stain. x33,600.
A reaction can be seen on a secretion granule (sg) in a cellular process (a), and on cellular processes (arrows) within the matrix (b and c).

Figure 45

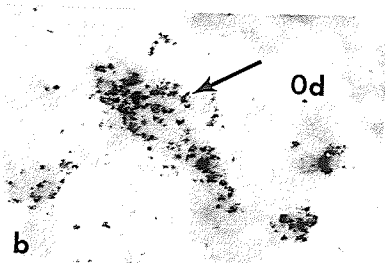
Electron micrograph of an odontoblast-like cell, 7 days following Adriamycin administration. Incubated in the presence of levamisole (medium 3) for 50 minutes. No contrast stain. x33,600.
A reaction is seen along the plasma membrane of the cell (arrowheads).



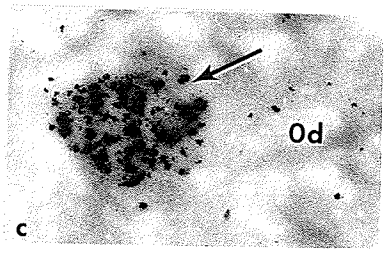
43



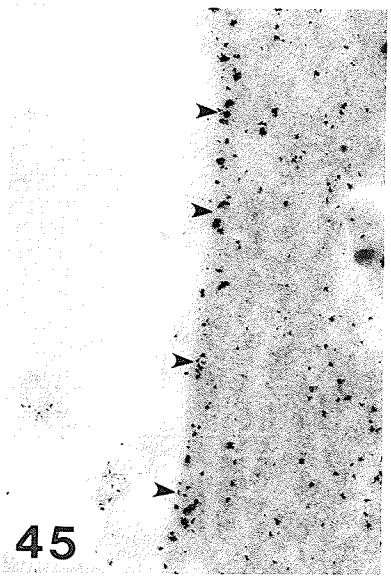
44a



b



c



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