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# THE ULTRASTRUCTURE OF DENTINE FROM RAT INCISORS FOLLOWING EXPOSURE TO SODIUM FLUORIDE AND POTASSIUM PYROANTIMONATE STAINING

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## Abstract

Weanling rats were given a single intra-peritoneal injection of sodium fluoride and control animals normal saline for four consecutive days. The fluoride produced a consistent response in the mineralizing dentine of the incisors in which a hypermineralized band was succeeded by a hypomineralized band. Potassium pyroantimonate staining for calcium ions showed that following injection of fluoride, in contrast to the controls, there were large amounts of calcium pyroantimonate in the pre-dentine and throughout the odontoblasts. This suggests that fluoride temporarily affects the membrane enzyme systems which maintain calcium concentration gradients between the odontoblasts and the matrix. The resultant influx of calcium is probably associated with the hypermineralization of the dentine matrix in which more hydroxyapatite crystallites are deposited. Upon recovery of the odontoblasts the matrix is relatively depleted of calcium resulting in matrix hypomineralization.

KEY WORDS: Ultrastructure, dentine, sodium fluoride, potassium pyroantimonate.

## Introduction

It is well established that in many species the exposure of developing dentine and enamel to a wide variety of ions, both in vivo, as a dietary additive or by subcutaneous or intra-peritoneal injection, and in vitro, produces alterations to the normal pattern of mineralization (Eisenmann and Yaeger 1969, Fejerskov et al. 1979) in that there is calcio-traumatic response (Irving and Weinmann 1948). This response varies considerably with the ions concerned but with fluoride is consistent and unique.

The response of dentine to subcutaneous injections of sodium fluoride, however, was first investigated by Schour and Smith (1934). In demineralized sections of dentine they described a first formed external band which stained with haematoxylin and eosin followed by a lightly eosinophilic staining band and an internal haematoxylin staining band. These observations were interpreted on the premise that the densely staining haematoxylin band previously contained more mineral so that a band of hypomineralization was followed by a band of hypermineralization. Earlier work contended that haematoxylin staining was not a reliable method for determining the degree of matrix mineralization in demineralized sections of various hard tissues (Cameron 1930, Hagens 1931) although its validity was later supported by Schour and Ham (1934) and by Irving (1943) in his work on the effects of sodium fluoride on dentine.

In studies of enamel mineralization using contact microradiography Applebaum (1943) supported these histological observations on the arrangement of bands. However, with the availability of high resolution contact microradiography (Yaeger and Eisenmann 1963, Osmanski and Yaeger 1964) for the examination of undemineralized ground sections the problem was resolved. The calcio-traumatic response to fluoride was clearly demonstrated as consisting of two bands, an external hypermineralized band and an internal hypomineralized band. Microradiography has shown that this response is consistent and forms at the time of injection (Yaeger and Eisenmann 1963, Yaeger et

al. 1964, Osmanski and Yaeger 1964, Eisenmann and Yaeger, 1969, 1972) is always paired, and that the degree of response is dose related (Yaeger and Eisenmann 1963). Furthermore it has been shown that the two bands of the response develop concurrently (Yaeger et al. 1964). The bands are not homogeneous and the hypomineralized band is often wider than the hypermineralized band.

Electron microscopy of the fluorotic rodent dentine (Yaeger 1963) demonstrated differences in electron density in relation to the level of mineralization. It was shown that the hypermineralized band contained more hydroxyapatite crystallites per unit volume which were of normal dimensions. The less electron dense hypomineralized bands, however, had crystallites of greater width than those in normal dentine. There were no differences in the organic matrix of hyper-, hypo- and normally mineralized sections, although subsequent polarized light studies suggested that fibres may be randomly arranged in the hypomineralized layers (Grady and Yaeger 1965). The examination of fluorotic dentine by Eisenmann and Yaeger (1972) and by Walton and Eisenmann (1975), however, showed irregular mineralization with discrete clusters of crystallites. The unmineralized matrix persisted in the vicinity of the odontoblast process. The hypermineralized dentine was not distinguishable in electron micrographs. With time normal dentine formed within the fluoride response areas so that only the unmineralized dentine persisted adjacent to the odontoblast process.

Tooth germs in organ culture have been used to determine the long and short term effects of fluoride on enamel and dentine formation and on the ameloblast and odontoblast (Bronckers et al. 1984a and 1984b). This system is more ideal to study the effect of fluoride concentration on the developing teeth and dental tissues since it is not possible to maintain constant levels in the serum because of the high clearance rates of this ion from the circulation (Larson et al. 1977). It was shown that concentrations of 2.6mM F were toxic for the enamel organ but had little effect on the cells of the dental papilla. In contrast to the ameloblasts, therefore, the odontoblast in organ culture exhibited little difference to the controls histologically (Bronckers et al. 1984a). At the ultrastructural level neither fluorotic odontoblasts or recovery odontoblasts in animals injected with fluoride showed any ultrastructural abnormalities (Walton and Eisenmann 1975). However, it has been clearly demonstrated in tissue culture that fluoride affects the metabolism of some non-collagenous components of dentine and in particular proteoglycan which has a reduced molecular size and altered charge mass distribution (Embery et al 1987).

The question that this study poses, therefore, concerns the role of the odontoblast in the utilization and transport of $Ca^{2+}$ to the mineralizing front since it is unclear whether the odontoblast is directly involved in this process (Heywood 1984). To this end the structure of dentine and the distribution of calcium in the odontoblasts of developing dentine exposed to high doses of fluoride was examined using the potassium pyroantimonate technique previously used in this laboratory (Appleton and Morris 1979a, 1979b, Morris and Appleton 1980, Morris 1981, Heywood 1984). This may help to understand the mechanisms by which the odontoblast controls the passage of calcium for matrix mineralization since many transferase enzymes require $Mg^{2+}$ as co-factors and are sensitive to the presence of fluoride (Embery and Smalley 1980).

## Materials and Methods

Two groups of ten black and white rats weighing approximately 100g were used. One group was given a single injection of 2.5% sodium fluoride in sterile distilled water (5mg/100g body weight) at the same time for four consecutive days and the other group was given an equivalent volume of normal saline. Both groups were sacrificed one hour after group one was given its final injection. The lower incisors were quickly extracted and the apical half divided transversely into slices 1 mm thick. Half the slices were fixed in 2.5% glutaraldehyde in cacodylate buffer pH 7.4 for 2-3h and half were fixed in potassium pyroantimonate osmium tetroxide solution at 4 $^{o}$C prepared according to the method of Appleton and Morris (1979a, b), Morris and Appleton (1980), and Morris (1981). All the tissue was then routinely processed and embedded in low viscosity resin (Spurr 1969) to facilitate adequate penetration.

### Contact microradiography

The apices of teeth fixed in glutaraldehyde and embedded in Spurr resin were sectioned transversally on an Isomet equipped with a 6μm diamond bonded wheel. Sections 150μm in thickness were X-rayed using a Machlett microradiography unit at 25kV and 5mA fitted with a copper target and a nickel window filter to provide a monochromatic beam of 1.84A selectively absorbed by hydroxyapatite. Exposure was for 8 min on high resolution plate type 1A (Kodak) which was developed at 1:8 dilution for 8min at 20$^{o}$C.

### Electron microscopy

Sections up to 5μm thick were used for purposes of locating areas of mature odontoblasts before thin sections were prepared on an LKB ultramicrotome using a diamond knife. Thin sections were mounted on copper or aluminium grids and examined in a JEOL 100CX Temscan system equipped with a Kevex detector together with a Link system 860 pulse processor.

### Energy dispersive analysis by X-rays (EDX)

For EDX sections approximately 1μm thick were coated with a thin conductive layer of carbon, using an Emscope sputter coater, and examined in a graphite holder. Analysis of the electron dense intracellular and extracellular

precipitates were undertaken in the STEM mode at 80kV for 200s with the holder tilted at 32° and a spot size of approximately 30nm.

## Results

### Contact microradiography

Microscopical examination of the X-ray plates showed that in the control animals there was no evidence of the mineralization of dentine having been interrupted by the injection of normal saline. In the test animals which received sodium fluoride injections for four days there were concentric bands of hyper- followed by relatively hypomineralized dentine (Fig. 1). These bands were about 15-20μm in width.

### Electron microscopy

The mineralized dentine exhibited a distinct and consistent response in the animals injected with sodium fluoride for four consecutive days. This is clearly evident in low power electron micrographs in which regular bands of different electron densities were present, the least electron dense being adjacent to the pre-dentine and the most electron dense deep in the mineralized dentine (Fig. 2). High power examination of these bands shows that differences in electron density are explained by variations in the numbers of crystallites present. In more electron dense areas the crystallites were more closely packed but there was no difference in crystallite dimensions (Fig. 3). Sometimes there was a narrow band of increased electron density in the mineralized dentine immediately adjacent to the pre-dentine (Fig. 2). Throughout the pre-dentine and dentine were the odontoblast processes and their numerous branches (Fig. 2). Within the pre-dentine large numbers of randomly orientated collagen fibres were present. Some of the coarse fibres, particularly those adjacent to the odontoblast process, were distinctive because of the electron dense precipitation clearly associated with the cross striations (Fig. 4).

There was a distinctive pattern of fine granular calcium pyroantimonate precipitation in the test animals and most significantly in those sacrificed immediately after the last injection of sodium fluoride. Although precipitate was present throughout the pre-dentine there were significantly increased concentrations associated with the plasma-membrane of the odontoblast process, the matrix adjacent to the odontoblast process and in the area adjacent to the junction of the odontoblast process with the odontoblast cell body. Precipitate was also accumulated in the inter-cellular spaces. There was also a significant amount of intra-cellular precipitate associated with the various secretory vesicles, and in particular the abacus bodies, both in the cell body and process as well as general background precipitation in the cytoplasm (Fig. 5).

In the control animals there was also markedly less pyroanatimonate precipitation in the pre-dentine overall and there was considerably less in association with the plasma-membrane of the odontoblast process and with the matrix around the odontoblast process. There was also less background intra-cellular pyroantimonate precipitation although large amounts of coarse precipitate were associated with intra-cellular organelles (Fig. 6). In the control animals there was little if any intra-mitochondrial precipitation but in the test animals fine precipitates were present in some mitochondria (Figs. 5,6).

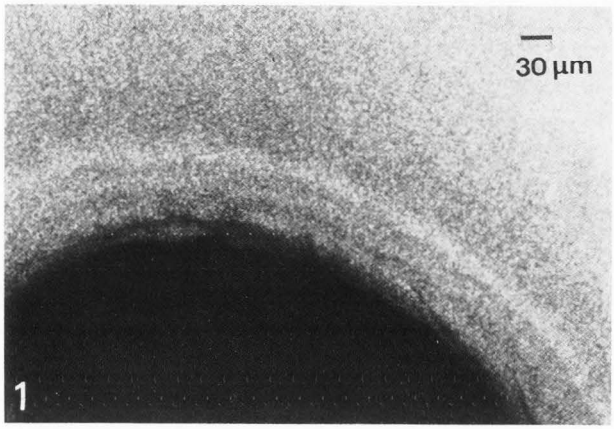


**Fig. 1** Contact microradiograph of labial dentine from lower incisor of test animal showing concentric bands of hyper- and hypomineralization approximately 15-20 μm wide.

### Energy dispersive analysis by X-rays

The results show a distinct calcium antimony peak with no evidence of any other cations being involved in the reaction. The same results were obtained for intra-cellular and extra-cellular precipitation (Fig. 7).

## Discussion

During dentine formation odontoblasts form a layer over the surface of the pre-dentine and are joined by junctional complexes both at the secretory and non-secretory ends of the cell. They are closely associated with the pre-dentine and the dentine via the odontoblast process (Jones and Boyde 1984). The work of Bishop (1985) using intravenously injected lanthanum as an electron dense tracer shows that this ion does not penetrate the odontoblast layer suggesting that calcium with a similar ionic charge and size probably behaves the same. Such evidence would suggest that there is a transcellular route for calcium into the pre-dentine but other earlier experimental results are ambiguous.

Using intra-peritoneal injections of Ca-45

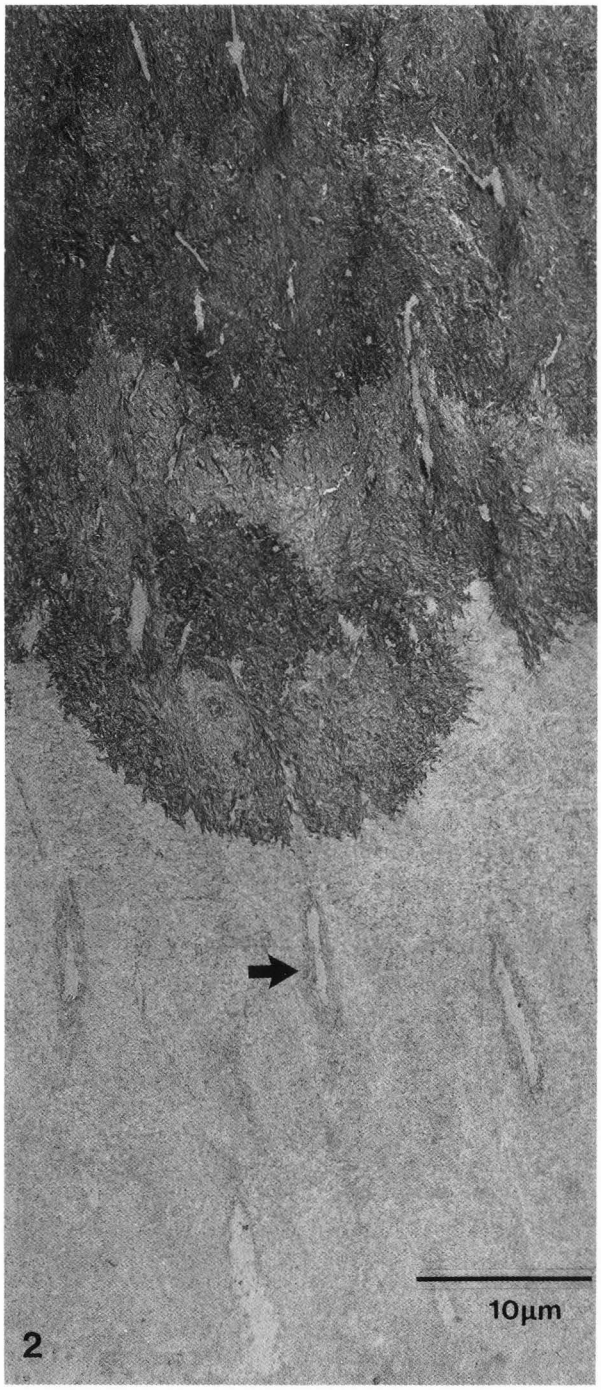


**Fig. 2** Electron micrograph of the mineralized dentine in the test animal. There are bands approximately 15-20 µm wide showing variations in the electron density associated with different levels of mineralization. The transition between bands is abrupt. In the pre-dentine there is fine granular precipitate particularly around the odontoblast process.

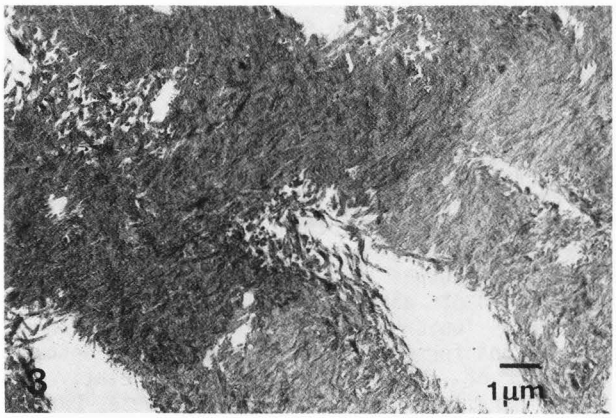


**Fig. 3** Junction between bands of different electron densities in the dentine of a test animal. This difference is related to the number of crystallites present.

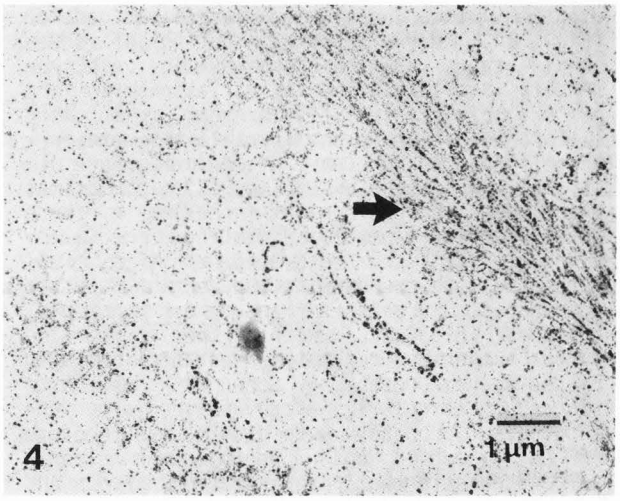


**Fig. 4** Accumulation of calcium pyroantimonate precipitate around the odontoblast process and related to the collagen fibres (→) in a test animal.

in rats Fromme et al. (1972) found activity progressively over the odontoblast, odontoblast process and mineralized dentine. Munhoz and Leblond (1974), however, recorded a rapid transfer of Ca-45 to the dentine pre-dentine junction and then to the dentine but there was no label associated with the odontoblasts. It was suggested therefore that the odontoblasts were not directly involved in the transfer of calcium to the mineralizing front.

A quantitative electron microscopical study of tooth germs by Nagai and Frank (1974)

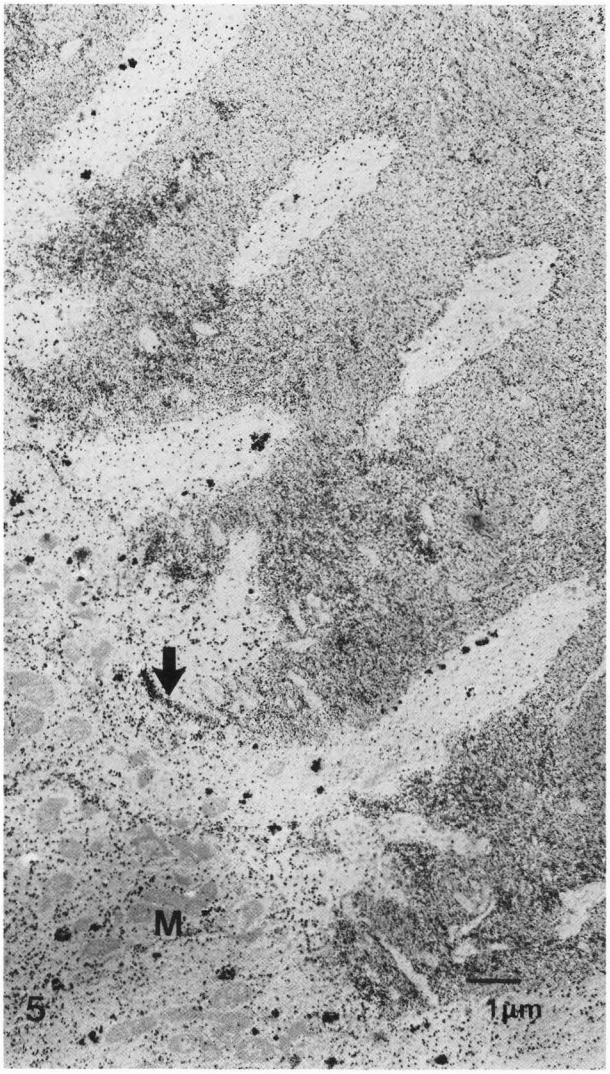


**Fig.5** The odontoblast and predentine in a test animal showing particularly dense accumulations of fine granular precipitate adjacent to the junction of odontoblasts and their processes. There is precipitate in the intercellular spaces (→) and in the background cytoplasm as well as associated with secretory vesicles. There is little precipitation in the mitochondria (M).

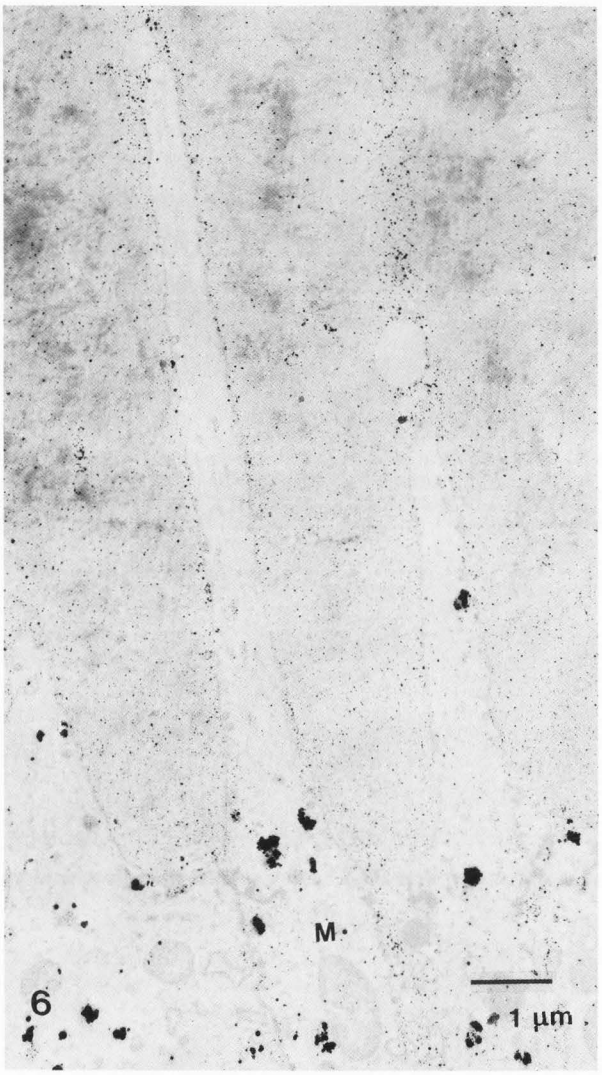


**Fig. 6** The odontoblast and predentine in a control animal. There is only a little precipitation within the odontoblast process and predentine. There is coarse precipitate associated with the secretory vesicles, little background precipitation and just a few granules in the mitochondria (M).

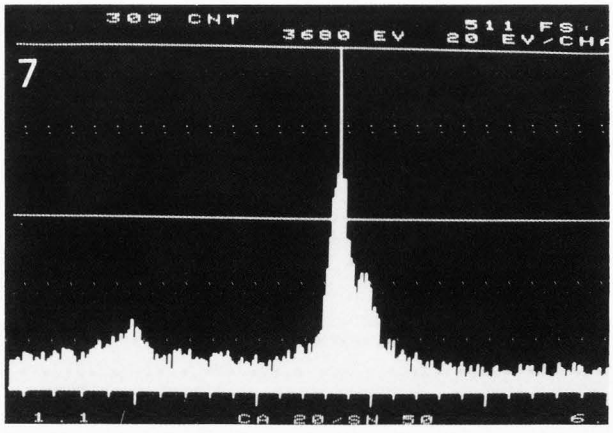


**Fig. 7** EDX analysis of precipitate showing peak for calcium pyroantimonate.

described two calcium pathways. A direct pathway through the inter-cellular spaces to the pre-dentine and dentine and an indirect intra-cellular route in which different cellular components were loaded and unloaded, but no Ca-45 was associated with secretory or coated vesicles (Frank 1979).

Studies on the odontoblast using the potassium pyroantimonate reaction to precipitate calcium as part of an electron complex have been undertaken by Reith (1976), Reith et al. (1977), Lyaruu et al. (1985), Kogaya and Furuhashi (1986), and in this laboratory by Appleton and Morris (1979a,b) and Heywood (1984). Using carefully controlled experimental conditions exchangeable $Ca^{2+}$ were localized in a variety of vesicular structures, particularly the abacus bodies, both in the odontoblast cell body and process. Furthermore electron probe analysis demonstrates a high concentration of calcium at the secretory pole of the odontoblast (Boyde and Reith 1977).

The consensus of the work described above is that the odontoblasts are directly involved in the utilization and transport of calcium, that is they regulate its passage to the mineralizing front. There is also strong morphological evidence in other mineralized tissue that the cells have a regulatory function with regard to the rate of calcium influx (Boyde et al. 1978, Boyde and Reith 1983). It is still possible, however, that this observed distribution is simply an expression of calcium homeostasis. Moreover it should be realised that micrographs merely represent static images of a dynamic situation.

There is no accurate information concerning the concentration of $Ca^{2+}$ in the sub-odontoblastic tissue fluids. However it is estimated that the extra-cellular concentration of $Ca^{2+}$ is at least a thousand times the intra-cellular level (Mulryan et al. 1964, Borle 1967). It is inevitable, therefore, that calcium will enter the odontoblast down the concentration gradient. Various mechansims are possible: passive diffusion, facilitated diffusion and agonist-sensitive facilitated diffusion. However since dentine formation shows rhythmic variations (Rosenberg and Simmons 1980) it is likely that the flow of calcium through odontoblasts is controlled by facilitated diffusion (Hohling and Fromme 1984) to coincide with periods of activity and rest. Intra-cellularly the calcium may then be transported to the various intra-cellular organelles and in particular the endoplasmic reticulum (Berridge 1987) or passed into the mineralizing pre-dentine or continually pumped against the concentration gradient back into the tissue fluids by the odontoblasts. Once inside the cell it is also well established that calcium uptake is a property of mitochondria (Chance 1965). However, there appears to be little uptake of calcium into the mitochondria of odontoblasts as demonstrated by the potassium pyroantimonate-osmium tetroxide reaction when compared, for example, with chondrocyte mitochondria (Morris and Appleton 1980) although intra-mitochondrial granules in odontoblasts have been demonstrated following freezing and freeze-substitution (Goldberg and Escaig 1984). Recently, however, it has been argued that this appearance is artifactual (Appleton 1987). From studies on other tissues, however, it has been demonstrated that the calcium binding of mitochondria varies considerably (Rubin 1982).

There are well documented ATP driven calcium pumps located in the plasma-membrane available for expelling calcium from the cell (Schatzman and Burger 1978, Carafoli 1984). In the odontoblast ATPase activity has not yet been found in the cell membrane. However an ATPase has been demonstrated in the intra-cellular vesicles which is activated by $Ca^{2+}$ and $Mg^{2+}$ (Linde and Granstrom 1978, Granstrom et al. 1978). Also there is an ATP dependent $Ca^{2+}$ concentrating ability in the microsomal fraction from rat incisor odontoblasts (Granstrom 1984). However it has recently been shown that this $Ca^{2+}$ buffering capacity is no different to that found in a variety of cells not involved in matrix mineralization (Lundgren and Linde 1987).

It is suggested therefore that the odontoblast layer forms and acts as a 'dentine membrane' in the manner of the 'bone membrane' proposed by Talmage (1969) and Simkiss (1975). According to their models dentine fluid in the pre-dentine is in equilibrium with the mineral phase and any excess calcium which leaks into this fluid is pumped out by the odontoblast. Calcium which enters the odontoblasts down the concentration gradient which exists between the odontoblasts and the sub-odontoblastic tissue fluids would likewise be pumped out by the cells.

If fluoride ions inhibit the transferease enzyme systems in the membrane (Embery and Smalley 1980) then calcium will pass into the pre-dentine without restriction until equilibrium is attained across the odontoblast layer. The large amount of fine granular precipitate in the pre-dentine matrix following the injection of sodium fluoride supports this hypothesis. The small precipitate particle sizes may indicate high calcium ion concentration and a rapid rate of precipitate formation since it is well established that the greater the degree of supersaturation the smaller the particles formed (Klein et al. 1972). This in turn will promote the formation of additional mineral nuclei and the formation of more crystallites as described by Yaeger (1963) to produce relative hypermineralization. Once fluoride has been cleared from the system then the membrane pumps will be reactivated but in the presence of low concentrations of calcium ions which will have been depleted producing relative hypomineralization.

These results must be considered in relation to the validity of the potassium pyroantimonate method for the accurate sub-cellular localization of exchangeable calcium ions. This technique was originally devised by Komnick(1962) for the sub-cellular localization

of sodium in soft tissues but has been since modified for the localization of calcium (Klein et al. 1972, Simson and Spicer 1975, Appleton and Morris 1979a,b, Wick and Heplar 1982, Kashiwa and Thiersch 1984, Mentre and Halpern 1988). The principal criticisms of this technique are that since aqueous media are involved there can be loss of translocation of ions and that the reaction may not always be specific for calcium. However, Klein et al. (1972) demonstrated that the precipitation threshold for Ca2+ with unbuffered 2% antimonate on ice at pH 7.8 is 10-6M compared with 10-5M for Mg2+ and 10-2M for Na+. The reaction with divalent cations is linear and even at the lowest concentration reaction efficiency is close to maximal. Also it is evident from apparent solubility products that not only does the initial precipitation take place at a lower concentration for calcium but, once formed, the calcium antimonate complex is unlikely to re-dissolve.

The presence of calcium in the odontoblast precipitates has been confirmed in earlier studies in my unit (Heywood 1984) and in this study by EDX. Furthermore, recent work suggests that potassium pyroantimonate may enhance calcium retention (El-Zainy et al. 1987). More importantly, there were clear and distinct differences in the distribution of calcium pyroantimonate precipitate between test and control animals which correlate with the changes observed in the mineralized dentine, that is, increased numbers of apatite crystallites and hypermineralized matrix following the administration of fluoride.

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## Discussion with Reviewers

**Reviewer 1**: It has been reported that fluoride affects the metabolism of some non-collagenous components of dentine. Phosphoryn, which is one of the non-collagenous proteins of dentine, is thought to be involved in regulating the deposition of mineral crystals in the matrix, and the root dentine contains only half the amount of dentine phosphoryn present in crown dentine, suggesting the differences in the mineralization process between crown and root dentine. Could you find any differences in the fluoride effects between labial and lingual dentine forming sites?

**Author**: There were no differences noted on the contact microradiographs. At the EM level it is difficult to be certain because of the small samples involved, but no differences were apparent.

**Reviewer 1**: Have you noticed any relationship of large amounts of antimonate reaction product following injection of fluoride with certain cell organelles of odontoblasts?

**Author**: Most of the organelles which contained precipitate in the controls contained more precipitate in the fluoride injected animals. The principal change following the injection of fluoride was the amount of non-specific intra-cellular precipitation and the large amount of fine extra-cellular precipitation associated with the junction of the odontoblast and the odontoblast cell body.

**Reviewer 2**: In your figures you are showing two types of PPA reaction products, a discrete lump type and a fine granular precipitate. Could you give a reason and significance for it?

**Author**: This is a common observation in the odontoblast where there are large numbers of intracellular vesicles containing these types of precipitate. The most likely explanation is that the fine precipitates are formed most rapidly, that is calcium ions are readily available for reaction with the antimonate complex, and the globular precipitates more slowly. It is apparent that the extra-cellular precipitates formed following the injection of fluoride are exclusively of the fine granular type and are, therefore, probably formed rapidly in the presence of high concentrations of calcium ions.

**Reviewer 2**: In the X-ray microanalysis the peaks of Ca and Sb overlap considerably. How did you confirm the presence of calcium in the antimonate reaction product?

**Author**: The results described in this study are an extension of work undertaken by myself and co-workers using PPA reaction to localize calcium in hard tissue forming cells. With the odontoblast work we have used calcium chelators to remove calcium and depolarizing solutions to remove ions such as Na which may precipitate with antimonate (Heywood 1984). Also we routinely compare the spectra obtained from the precipitates with spectra from embedded potassium pyroantimonate. This rapidly demonstrates the replacement of the potassium peak at 3.34 keV by the calcium peak at 3.69 keV producing the characteristic spectra seen in Fig. 7.

**Reviewer 2**: Does the PPA technique show bound and unbound Ca in the tissues?

**Author**: It is unlikely that unbound Ca is precipitated since at 10-6M (Hohling and Fromme 1984) it represents only a very small fraction of the total concentration of calcium ions. This is below the threshold for reacting with antimonate and is probably lost during tissue processing. The random precipitation seen following the administration of fluoride probably represents an increase in the amount of free calcium entering the cell.

**Reviewer 2**: Do you think shutting off the fluid flow from dentine will effect enamel formation?

**Author**: Dentine formation occurs in advance of enamel formation so that dentine is present first at the site of the future amelodental junction. If this is first formed dentine contributes mineral ions to the initial layer

of enamel then any interference with dentine formation will affect this enamel.

**Reviewer 3**: Is the fixation and staining in potassium pyroantimonate osmium quick enough to provide arrest of calcium in its original location in the cell and matrix?
**Author**: Since its introduction as a cytochemical technique the potassium pyroantimonate method for the localization of calcium has undergone numerous modifications in an attempt to optimise the results for specific tissues. These changes have involved altering the fixative, including buffer, and or altering the concentration of the pyroantimonate solution. Osmium tetroxide has been the fixative of choice since it penetrates tissue more rapidly than glutaraldehyde and allows the fast passage of pyroantimonate across cell membranes. Fixation and precipitation, therefore, should be concurrent events resulting in an accurate picture of calcium distribution. Osmium fixatives however by removing lipid from membranes make them freely permeable to small ions and protein molecules. We measured the percentage loss of Ca45 from condylar cartilage of rat mandible during fixation with potassium pyroantimonate osmium and processing at 2.95% compared with 9.47% for primary glutaraldehyde fixation and post osmication (Morris 1981). It could be argued, therefore, that a large proportion of the precipitable calcium retains its original location.

**Reviewer 3**: Is it possible that some of the precipitation product may be lost during ultramicrotomy when water is used as a trough liquid?
**Author**: According to Klein et al (1972) the precipitate threshold for $Ca^{2+}$ with unbuffered 2% antimonate on ice is much lower than for other physiological cations and its apparent solubility product such that it is unlikely to redissolve.

**Reviewer 3**: You suggest that fluoride causes changes in calcium distribution by temporarily affecting the membrane enzyme system responsible for maintenance of calcium concentration gradients between odontoblasts and dentine matrix. Are there any other mechanisms, such as for example effects of fluoride on the polymerization of cytoskeletal proteins and thereby on endo and exocytosis?
**Author**: At the concentrations used fluoride affects numerous energy dependent enzyme systems and it is possible that polymerization of cytoskeletal proteins would be affected.