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ANALYSIS OF EARLY HARD TISSUE FORMATION IN DENTINE BY ENERGY DISPERSIVE X-RAY MICROANALYSIS AND ENERGY-FILTERING TRANSMISSION ELECTRON MICROSCOPY

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Abstract

Thin cryosections and sections of embedded tissue were prepared from dentine of cryofixed rat incisors. Energy dispersive X-ray microanalysis (EDX) and electron energy-loss spectroscopy (EELS) have been applied to study the calcium and phosphorus distribution in predentine of these incisors. A small enrichment of calcium and phosphorus was found in the predentine zone near the dentine border.

Element distributions were correlated with analyses of the early crystal formation in dentine. These investigations were carried out by parallel applications of electron spectroscopic diffraction (ESD) and electron spectroscopic imaging (ESI) using zero-loss filtering. It was found that the earliest crystal formations already showed the lattice of the hexagonal mineral apatite. They form parallelly arranged chains of dots which coalesce rapidly to form "needle-like" crystallites along the collagen microfibrils.

Key Words: Electron probe X-ray microanalysis, electron energy-loss spectroscopy, electron spectroscopic diffraction, cryosection, calcium, phosphorus, predentine, dentine, calcification, mineralization.

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Introduction

In the developing dentine, the long prismatic odontoblasts form a collagen rich extracellular matrix between the odontoblasts and the mineralized dentine tissue, the predentine. Mineralization happens at the predentine/dentine border of the continuously growing rat incisor. Up to now, the rat incisor has been well investigated and often used as a model for studying hard tissue mineralization.

The dentine mineral apatite consists mainly of calcium and phosphorus so that the distribution of these elements in predentine and especially at the mineralization front seems to be helpful to get information concerning the manner of mineralization. Quantitative electron microprobe analyses of predentine were first done by Höhling *et al.* (1967) and later by Ashton *et al.* (1973), Nicholson *et al.* (1977), Höhling *et al.* (1991) and others. They used cryosections in a thickness range of 4-8 μm and semi-thin sections of shock frozen dentine tissue embedded in resin. Several disadvantages of these specimen types have to be taken into account. On one hand, cryosections in this thickness range show poor morphological structure in the electron microscope, and microprobe analyses have a low spatial resolution. So it is often difficult to relate elemental concentration to morphology. On the other hand, resin infiltration may cause elemental dislocations in resin sections.

Furthermore, spatial resolution of energy dispersive X-ray microanalysis (EDX) of several μm thick sections is not sufficient to resolve the border of the mineralizing zone due to electron scattering. Therefore, increasing use is made of electron energy-loss spectroscopy (EELS) of transmitted electrons for investigations of biological specimens particularly near the mineralization front of dentine tissue (Blottner and Wagner, 1989; Plate *et al.*, 1992). This can be achieved by using the scanning mode in a dedicated scanning (STEM) or in a transmission electron microscope (TEM) with a spectrometer behind the camera chamber or by using an imaging filter lens in the column of the TEM.

In this study, it was possible for the first time to compare the results of our widely used dry sections of shock frozen, freeze dried, and embedded tissue with

those of ultrathin cryosections, obtained with a Reichert cryoultramicrotome. New information was obtained by a fruitful combination of several techniques. We obtained quantitative elemental contents using EDX. For high spatial resolution of elemental distribution, electron energy-loss spectroscopy (EELS) was used. Early crystal formations in dentine were analyzed by parallel applications of electron spectroscopic imaging (ESI)- and electron spectroscopic diffraction (ESD)-mode. Zero-loss filtering was used to enhance contrast.

The intention of this paper is to make a contribution to the understanding of the prestages and early stages of the calcium-phosphate formation in predentine and early dentine.

Material and Methods

Rat incisors were obtained from young Wistar rats with a weight of about 150 grams. Directly postmortem, the continuously growing incisors were isolated and transferred to liquid nitrogen-cooled propane for cryofixation as described previously (Plate *et al.*, 1992). Tissue preparation and cryofixation took less than three minutes.

In the first series of experiments, the incisors were freeze-dried at -80°C under vacuum in an Edwards-Pearse tissue dryer (preparation of dry resin sections reviewed in: Barckhaus and Schmidt, 1991). The freeze-dried tissue was then infiltrated with chlorine free Spurr's resin, which was polymerized at 65°C. For EDX analyses, dry sections in a thickness range of 250 nm were cut with a diamond knife on an ultramicrotome and were transferred to carbon-coated double-folded grids. Analysis by energy-filtering TEM (EFTEM) required ultrathin sections which were in a thickness range of 50-90 nm for these measurements. The sections were cut with a diamond knife on an ultramicrotome and were transferred onto 300-mesh copper grids. The water-contact of these ultrathin sections was reduced to only a few seconds to minimize dissolution of crystallites by water.

Second, cryosections with a thickness of 80-100 nm were prepared with a Reichert FC4-Ultracut cryoultramicrotome and transferred from the dry glass knife onto a Pioloform and carbon-coated 75-mesh copper grid by using an eye lash. Then the ultrathin sections were freeze-dried. The preparation of cryosections was described in detail by Zierold (1986, 1991).

The analyses were carried out with a Philips TEM 301 with STEM attachment, equipped with a Si(Li)-detector and an EDAX system (9100) for EDX microanalysis. The accelerating voltage was 80 kV and the tilt angle between section and detector was 30°. Usually X-ray spectra were obtained by using a scan raster of 1 x 1 μm^2 and a counting live time of 100 seconds. For quantification, the analytical continuum-method of Hall (1971) and Hall *et al.* (1973) was used.

All quantitative results are given in weight-percent per dry mass (g/100g). The Ca/P ratios are given as weight ratios.

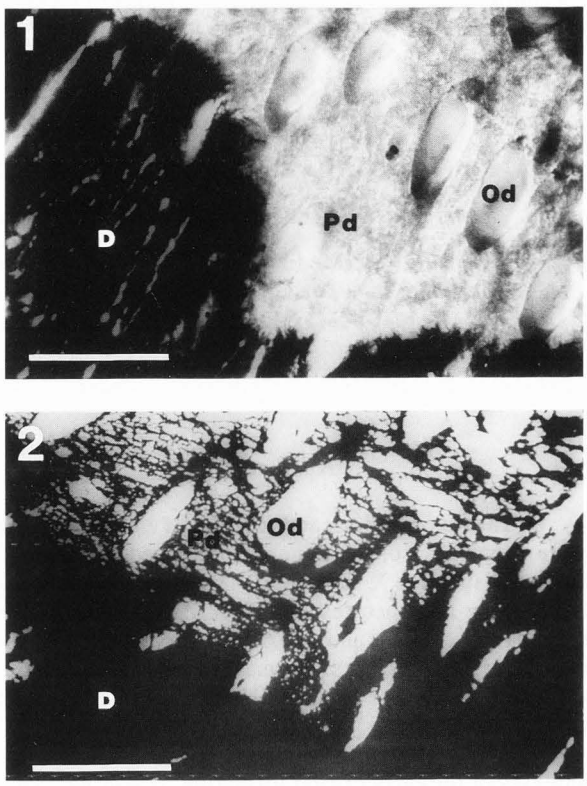


Figure 1. STEM image of developing dentine in rat incisor near the apex; semi-thin dry section of shock-frozen, freeze-dried, embedded tissue, section was not cut perpendicular to the long axis of tooth. D = Dentine, Pd = predentine, Od = Odontoblast process. Bar = 5 μm.

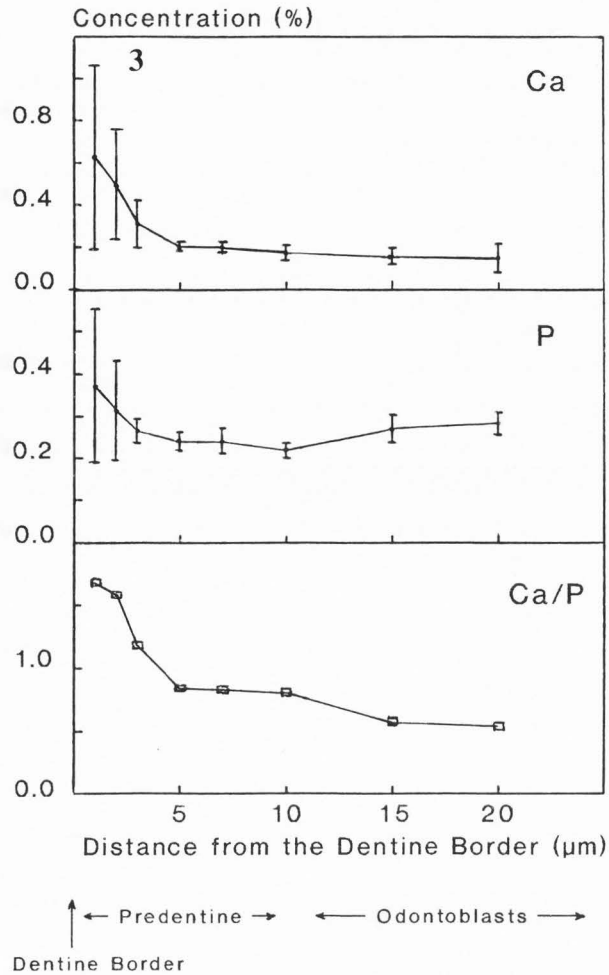
Figure 2. STEM image of the ultrathin cryosection of the same region as shown in Fig. 1. D = Dentine, Pd = predentine, Od = Odontoblast process. Bar = 5 μm.

The measurements by EFTEM were carried out in the Zeiss EM902 operated at an acceleration voltage of 80 kV. In conventional TEM, the scattering contrast is generated by the decrease of the number of electrons that pass through the objective aperture and contribute to the image. In order to increase the contrast and to improve the resolution, zero-loss filtering was used in the ESI-mode and also in the ESD-mode to get diffraction patterns with low background.

Results

Figures 1 and 2 show a resin section and a cryosection of the incisor tissue. All analyses were carried out on cryofixed rat incisors. The 'Ghosts' of ice crystallites were normally in a diameter range below 0.5 μm, only occasionally reaching the 1 μm range.

EDX analyses of dry resin sections (Fig. 3) show low calcium contents of about 0.2 % in the odontoblasts and in predentine with a clear increase to the dentine



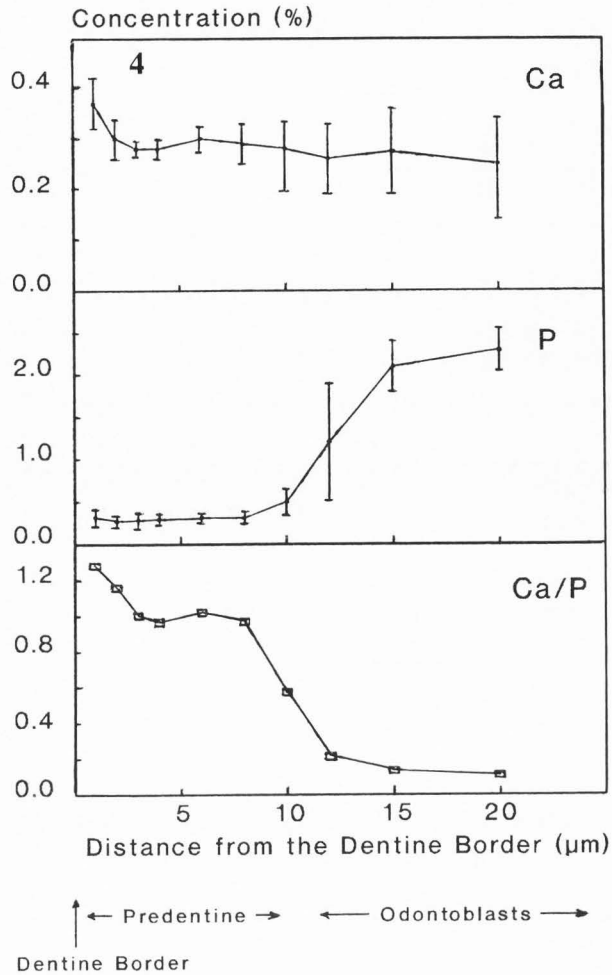


Figure 3 (at left). Plots of Ca and P concentrations, and the Ca/P ratio against the distance of the scanning area from the mineralization front of resin sections. EDX analyses were applied, concentrations are given in %/dry mass (mean ± standard deviation, SD), number of measurements at each point is higher than 25. Notice: predentine is between the mineralization front at 0 µm and the odontoblast layer which appears at about 12 µm.

Figure 4 (at right). Ca and P concentrations, and Ca/P ratio in ultrathin cryosections. Measurements and plots were done as in Fig. 3. At 20 µm, the number of measurements was 12, at other distances number was higher than 20.

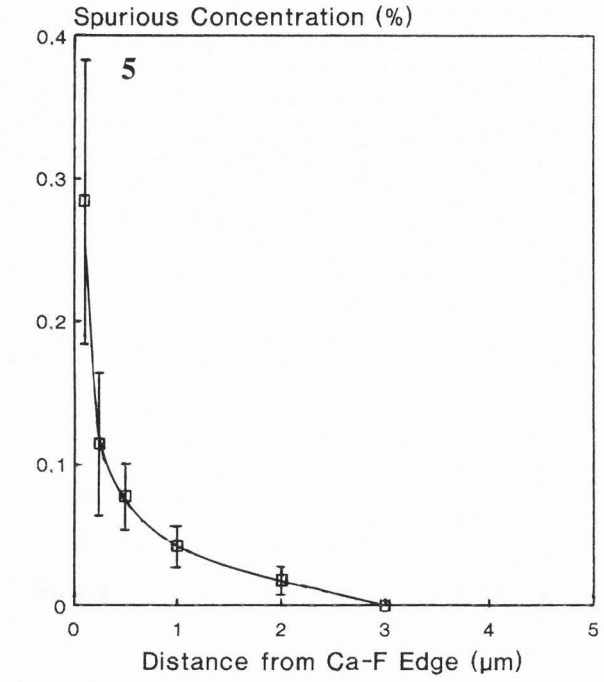
border starting at about 3 µm in front of the border. In the odontoblasts, phosphorus concentrations are slightly higher than in the beginning region of predentine. Similar to calcium, phosphorus shows an increase at the dentine border. But since the phosphorus increase is lower than that of calcium, the Ca/P ratio increases as well.

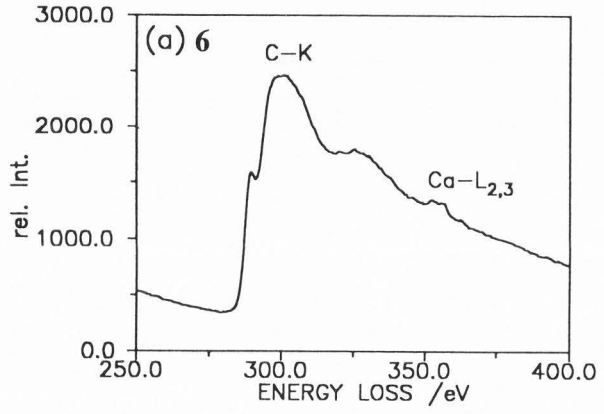
Figure 4 shows the EDX results of cryosections for calcium, phosphorus and the Ca/P ratio. The calcium content is about 0.3% at the odontoblasts and in the predentine. Only at a distance of 1 µm from the mineralization front the calcium concentration shows a small increase. There is a high phosphorus content in the odontoblasts which changes to concentrations in the range of 0.3% at the predentine. A very small increase of phosphorus at the mineralization front is visible. Furthermore, we have found areas of higher phosphorus content in the predentine which are not included in Fig. 4. These areas were not only located at the mineralization front. The Ca/P ratio is about 1 in predentine and increases slightly to 1.3 near the mineralization front.

Sections (0.5 µm thick) of an embedded CaF_2-layer were taken as a model to estimate the spurious calcium concentration in predentine obtained at the dentine border (Fig. 5).

EELS spectra of resin sections (Fig. 6) and ultrathin cryosections (Fig. 7) show a slight increase of the calcium content in predentine near the dentine border and a sharp increase at the early dentine crystallites.

Figure 8a shows ESD patterns of the earliest mineralized dentine crystallites with less and more diffuse Debye-Scherrer rings than in more mature crystallites (Fig. 8b). ESI-mode images with high magnification





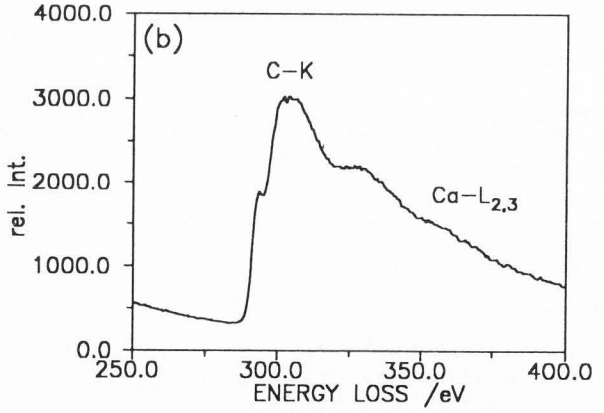


Figure 6. EELS spectra in predentine of embedded rat incisors (thickness range of the sections about 60-80 nm): **a**) directly at the dentine border, and **b**) in the middle of predentine show the C-K edge and the Ca-$L_{2,3}$ lines.

Figure 5. Spurious calcium concentration against distance to a CaF_2-border. The spurious calcium content is caused by secondary excitation of calcium atoms in the CaF_2-layer. CaF_2-layer was embedded in Epon, dry sections were 0.5 μm thick.

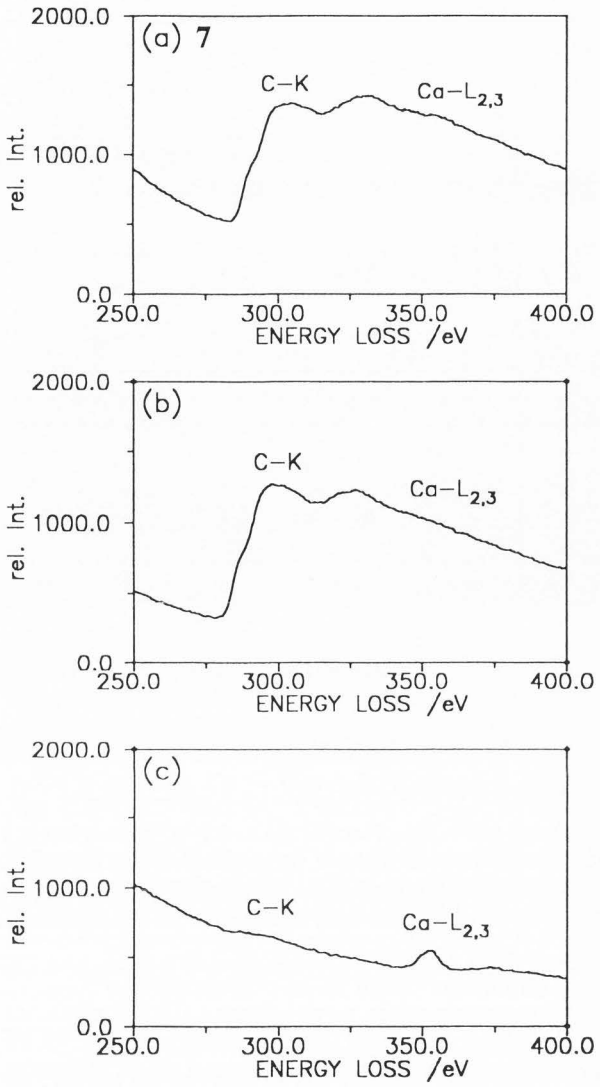


Figure 7. EELS spectra in predentine of ultrathin cryosectioned rat incisors (thickness range about 80-90 nm): **a**) directly at the predentine/ dentine border; **b**) in the middle of the predentine show and **c**) directly taken from the first dots at the C-K edge and the Ca-$L_{2,3}$ lines.

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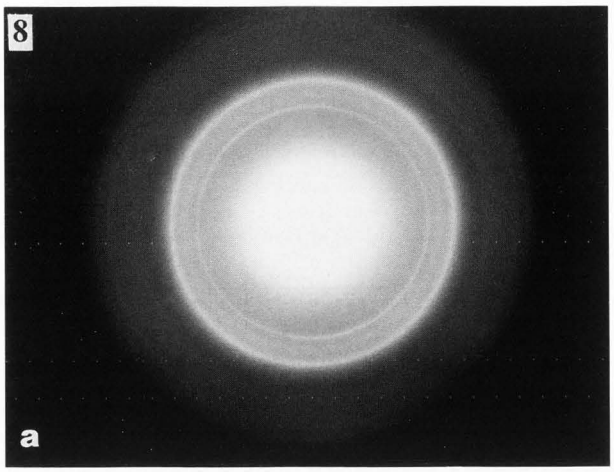
show early crystal formations at the dentine border which can be described as chains of "dots" coalescing to "needles" (Fig. 9).

Discussion

In order to prevent postmortem elemental displacements, the incisors were cryofixed as rapid as possible. In spite of the rapid cooling ice crystals develop,



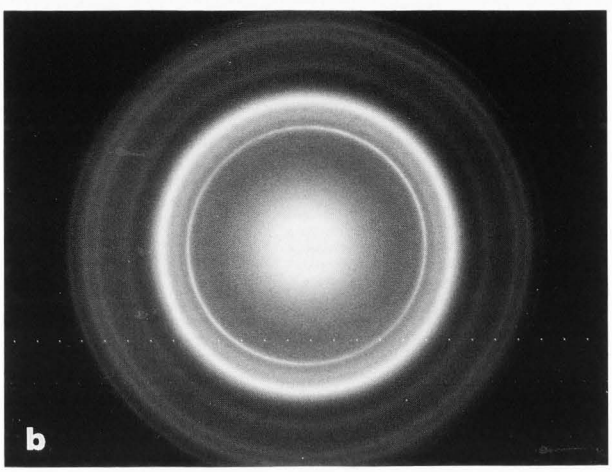


Figure 8 (at left). ESD-patterns of **a**) the earliest mineralized dentine crystallites (dot region); and **b**) from more mature crystallites in dentine. Zero-loss filtering was used.

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at a depth of about 10 μm from the tissue surface, and cause damage of the tissue and a slight displacement of unbound elements. Since the predentine is surrounded by dentine which is covered by enamel or cementum, the distance to the tissue surface lies in the mm range so that ice crystal formation must be accepted. Echlin (1991) reported that ice crystallites in a bulk sample have a diameter range of 0.2-2.0 μm. After freeze-drying, ice crystals appear as 'ghosts' with a diameter up to 1 μm. Taking this situation into account, sufficiently large scanning areas, at least 1 x 1 μm^2, have been chosen in order to get a good average value for the local elemental content.

An advantage of dry resin sections is that the tissue structure seems well preserved (Fig. 1). Additionally, embedded tissue is quite stable and therefore easy to handle. However, during the process of resin infiltration and before its polymerization, diffusible ions or small molecules may be displaced. In ultrathin cryosections, elemental displacements are also possible. Von Zglinicki and Zierold (1989) have pointed out that the exposure of freeze-dried cryosections to air can result not only in loss of image contrast but also influence elemental distribution. However, in general, elemental distribution is certainly much more influenced by the process of resin embedding of shock frozen tissue than by cryosectioning. Moreover, the morphology of dentine tissue is well enough preserved in ultrathin cryosections; so it is possible, e.g., to observe the odontoblast processes in scanning electron images (Fig. 2). Extra- and intracellular regions could be distinguished and consequently analyzed separately. Sections, cut perpendicular to the long axis of the incisor from the middle to the apical regions, have been analyzed.

Elemental analyses of resin sections and cryosections

Results of our EDX analyses of dry resin sections are in rather good agreement with earlier investigations of resin sections. Höhling *et al.* (1972) reported that calcium and phosphorus contents in predentine are in a range of 0.2-0.6% and 0.2-0.7%, respectively. Nicholson *et al.* (1977) pointed out that there are areas, with a second stage of Ca-enrichment near the mineralization front, which are in rather good correlation with the high standard deviation near the mineralization front in Fig. 3. Recently Höhling *et al.* (1991) have found an increase of the Ca-K_α X-ray counts near the dentine border.

Earlier investigations of thick cryosections have found elemental contents in the range of 0.2-0.4% for Ca and 1-4% for P (Höhling *et al.*, 1968), 0.1-1.3% (mean 0.28%) for Ca and 0.3-3.4% for P (Ashton *et al.*, 1973) and up to 4% for Ca in enriched zones (Nicholson *et al.*, 1977).

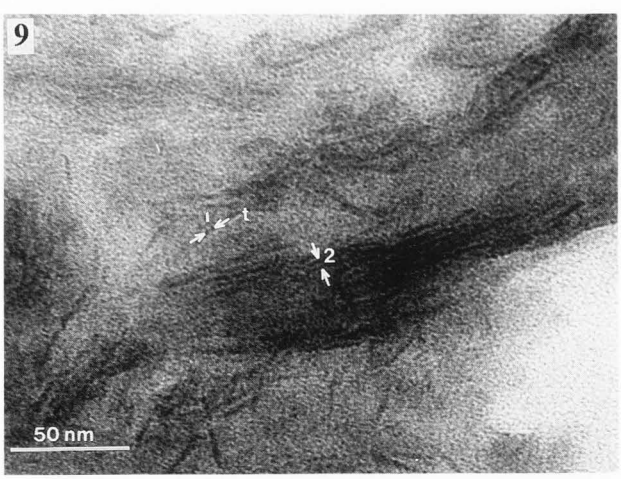

Figure 9. Earliest crystal formations can be described as mineral chains of dots (arrows 1 designate the dot to dot distances) along the collagen-microfibrils which rapidly coalesce to form parallel arranged "needle-like" crystallites in which the dots are no more visible (arrows 2 designate the lateral center to center distances).

Generally, the previously found phosphorus contents of predentine were higher than our contents found in ultrathin cryosections. The main reason for this difference might be the different thickness levels of the sections. In thick cryosections, it is nearly impossible to differentiate between the predentine near the odontoblasts and the odontoblast region. Therefore, the reported high phosphorus values might be partly due to intracellular regions which have been analyzed together with extracellular regions in the thick sections.

EDX results of ultrathin cryosections compared with resin sections show some evident differences. For calcium and phosphorus, the mean concentrations in predentine is slightly higher in cryosections than in resin sections. Further, there is a stronger increase of both elements towards the mineralization front in resin sections. The phosphorus content measured in the odontoblasts is nearly ten times higher in cryosections than in resin sections.

The difference of measured phosphorus contents in the odontoblasts between cryosections and resin sections gives an impressive example how resin infiltration may cause element displacement. This difference demonstrates a prominent preparation artefact. In cells, only a small portion of phosphorus is found as inorganic phosphate; so it seems that even organic phosphate molecules were displaced by the resin. Concerning the increase of calcium and phosphorus at the mineralization front, which appears especially in resin sections, a diffusion of these elements from the dentine border into predentine seems conceivable. Diffusion is only possible if mobile components of calcium and phosphorus occur at the mineralization front. Such mobile components can be calcium ions, phosphate ions or small molecules of phospho-proteins which are not bound to the matrix.

Calcium contents found in ultrathin cryosections are in good correlation to earlier results except some high contents near the mineralization front (Nicholson *et al.*, 1977). It is necessary to discuss whether the increase of calcium near the dentine border, which was also found in ultrathin cryosections, is a real increase, or due to a secondary excitation of calcium atoms in the neighboring dentine, or due to a preparation artefact. Felsmann (1987) has analyzed the question of secondary excitation at sharp gold edges embedded in Epon. Different factors which influence the value of secondary excitation were found, and according to his results, Felsmann has given an approximation for the distance from a calcium edge for which the secondary excitation of calcium is detectable. He has shown that for a section thinner than 1 μm, secondary excitation starts at a distance of 5 μm. To get an estimation for the amount of the spurious calcium content in predentine near the dentine border, which is stimulated by secondary excitation, a model was prepared. Figure 5 shows the spurious calcium content in a 0.5 μm thick section plotted against the distance to a CaF_2 edge embedded in resin. The spurious calcium concentration at a distance of 1 μm to the edge is about 0.05%. According to this model, the spurious calcium content in predentine would roughly be estimated as being below 1% at a distance of 1 μm to the mineralization front. Therefore, the calcium gradient of the resin sections cannot be caused only by secondary excitation of calcium. As discussed before for resin sections, this gradient may also be caused by a preparation artefact. It is not possible to use the approximation for cryosections because they are only 100 nm thick and are not embedded. Because of this, the spurious calcium content in ultrathin cryosections must be lower or even not detectable. Finally, EDX analysis of ultrathin cryosections indicates a slight calcium increase at the mineralization front. However, influences of secondary excitation cannot be excluded.

Consequently, EELS with a spatial resolution of 10 to 100 nm was applied to clarify the calcium increase close to the mineralization front. In ultrathin resin sections, the calcium content, which was measured in predentine directly at the dentine border, was higher than the calcium content in the middle of the predentine zone (Figures 6a and 6b). Also, EELS spectra of ultrathin cryosections support the existence of an increase of calcium concentration at the mineralization front. Figure 7b shows an EELS spectrum at a distance of about 2 μm to the mineralization front and Figure 7a is directly taken besides the earliest crystallites. The calcium content rises at the region of the earliest crystallites; therefore, the calcium concentration is high in an area which includes a few dots (Fig. 7c).

Earliest crystal formations in dentine

The early crystal formation, just at the dentine border, can be examined by parallel applications of electron spectroscopic diffraction (ESD) and electron spectroscopic imaging (ESI). Zero-loss filtering in the ESI-mode increases the scattering contrast and improves the resolution because the chromatic aberration caused by inelastically scattered electrons in the specimen is avoided (Reimer, 1991). Zero-loss filtering was also used in the ESD-mode for removing the inelastically scattered electrons, contributing to the background, from the elastically scattered electrons to get clear diffraction patterns (Reimer *et al.*, 1990).

The ESD patterns of the crystallites contain only Debye-Scherrer rings of the hexagonal mineral apatite. Figures 8a and 8b show that the earliest crystal formations have more or less diffused Debye-Scherrer rings than the mature crystallites. Therefore, it can be concluded that the early crystallites still contain some lattice defects which are annealed out to some extent with crystal growth (Plate and Höhling, 1992).

In the ESI-mode, the earliest crystal formations exhibit chains of dots which rapidly show a "needle-like" character (Fig. 9). We assume that the dots develop in polar regions of collagen-microfibrils where calcium and phosphate groups are concentrated at polar amino acids and result in crystal nucleation. The center-to-center distances between the dots have the same distance range as the distances between the collagen cross-bands. So it seems that the crystal nuclei develop at charged amino acids, so called "active sites". Further, the center-to-center distances between the parallel arranged chains of dots or needles (Fig. 9) are in the same range (3.5-5.0 nm) as the center to center distances between the collagen-microfibrils ($\approx$ 4.0 nm) (Raspanti *et al.*, 1989). The "needles" can coalesce rapidly in lateral directions to form "ribbon-plate-like" crystallites. In a characteristic difference to the collagen mineralization of other hard tissues (bone) and also to physiological mineralization of turkey tibia tendon, the mineral

strands in dentine show only rarely the characteristic macroperiod of the collagen fibrils (67 nm) (Boyde 1974; Höhling *et al.*, 1990).

Conclusions

Generally, in the predentine, the calcium and phosphorus concentration is much higher than necessary for *in vitro* mineralization of collagen rich systems. Therefore, a mechanism which inhibits mineralization in the predentine is to be expected. Fujisawa *et al.* (1987) showed that soluble phosphoproteins inhibit precipitation of calcium phosphate *in vitro*. On the other hand, an induction of mineralization by immobilized phospho-proteins was demonstrated recently (Glimcher 1989; Linde *et al.*, 1989). The Ca-P data presented in this paper may be taken as a suggestion that there is a small transition zone between predentine and dentine at the dentine border. In this "turnover zone", calcium can be bound, perhaps electrostatically, to the "active sites" of the collagen-microfibrils, and also the immobilization of phospho-proteins by binding to the matrix seems to be possible.

Acknowledgements

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Discussion with Reviewers

W.A.P. Nicholson: The technique of estimating spurious concentrations of Ca using a model system is well known (Nicholson and Dempster, Scanning Electron Microsc. **1980**;II:517) and is sensibly applied to this system for EDX analysis. Have the authors any data from a similar experiment using EELS which would illustrate the difference in spatial resolution of the two analytical techniques?
Authors: There are some interesting comparisons between EDX and EELS analyses of biological specimens but we know of no study which illustrates the difference in spatial resolution. Weiss and Carpenter (1992, Ultramicrosc. **40**, 339) have reviewed experimental parameters which limit the spatial resolution and sensitivity of parallel EELS in a STEM; they reported that in the sub-nanometer range, the spatial resolution of the scanning probe technique cannot be described only by the probe diameter. However, the Zeiss TEM 902 EELS-system is different from that of the STEM, so that the spatial resolution might also be different.

G.M. Roomans: The authors assume that the difference between Figures 3 and 4 is due to displacements of elements by the resin embedding. First of all, the authors do not take into account that compared to a freeze-dried cryosection, a section of freeze-dried resin-embedded tissue will have lower concentrations for all elements, simply because the mass of the resin is added to the freeze-dried tissue and "dilutes" the elements present. Different amounts of resin may be present at different sites in the sample, depending on the density of the tissue. Secondly, displacement of phosphorus bound to macromolecules by resin is highly unlikely. Thirdly, most groups working with freeze-dried embedded material report only small losses, even of diffusible ions.
Authors: First, usually there are three ways [weight per cent (of the specimen), weight per cent per dry mass and weight per cent per wet mass] used to give the elemental concentration of an EDX analysis. In freeze-dried cryosections, weight per cent and weight per cent per dry mass are the same; and in resin sections with embedded soft tissue, the nominal weight percent is often comparable to weight per cent per wet mass because the water of the tissue is mainly replaced by the resin of which Z^2/A (Hall equation) is rather similar to the Z^2/A of water. So we had to take into account the Pioloform for both kinds of sections and the resin content (assumed equal to water content) for the resin sections only. In our study, we have used different types of standards agar-agar-standards (Barckhaus *et al.*, 1991, Inst. Phys. Conf. Ser. No. 93, Vol. 3, 573) and organic standard components in resin (unpublished). Also some elemental measurements on cryosections using dextran standards were in good agreement with the other results. Of course, we have noticed that the weight per cent for cryosections is generally different from the weight per cent of resin sections. However, it is difficult to compare the elemental concentrations of cryosections with those of resin sections, because indeed, we could not take into account different amounts of resin at different sites of the embedded tissue and also the behavior of the two kinds of the sections under the electron beam may be different.

Secondly, it is difficult to discuss the displacement of phosphate. We agree, assuming that phosphate being bound to the predentine matrix or large, indiffusible macromolecules (e.g., proteoglycans), does not get displaced. However, a displacement of phosphate bound to small macromolecules, (e.g., phospho-proteins) seems to be possible.

Thirdly, we think a special study is necessary which investigates element displacements in several biological tissues. Such a study should systematically compare the elemental distribution of ultrathin, freeze-dried cryosections with that of freeze-dried, resin sections of embedded tissues.

J. Wroblewski: We have recently shown that even a short contact with water during cutting procedure causes loss and redistribution of Ca (Wroblewski J. *et al.*, 1991, Scanning Microsc. **5**, 885). How did you verify that no loss of calcium occurred during cutting for EELS?
Authors: Certainly, there is a loss of calcium during the cutting process even if the contact with water is short. Therefore, some errors in the EELS measurements might exist. This was discussed by Plate *et al.* (1992).

J. Wroblewski: The standard deviations shown in Figures 3 and 4 are rather high. Can the changes in the changes in the Ca/P ratios be significant?
Authors: In the small region near the mineralization front, we expect remodeling- and turnover-processes; therefore, in the direction of crystal nucleation, the standard deviations should be high. Further, standard deviations might rise up, because at same distances to the dentine border, different presteps of mineralization may exist. However, the mean Ca/P ratio increases significantly closer to the dentine border.

J. Wroblewski: In Figures 1 and 2, the odontoblast processes can be clearly distinguished. Have you tried to analyze separately the elemental content of the cells and the predentine between the cells?
Authors: Yes, we have. In ultrathin resin sections, with water contact, nearly all elements of the odontoblastic processes have been lost. In dry resin sections (no water contact), the contents of phosphorus and calcium in the odontoblastic processes were similar to contents in predentine. In ultrathin cryosections, the phosphorus content in the odontoblastic processes could reach 2%.

J. Wroblewski: How do you explain that only the distribution of phosphorus was affected by the embedding procedure?
Authors: Calcium may also be affected by the embedding procedure, however, its concentration in the predentine is not much different from its concentration in the odontoblasts; so the effect is not evident.