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Bovine and equine peritubular and intertubular dentin

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Abstract

Dentin contains 1–2 μm diameter tubules extending from the pulp cavity to near the junction with enamel. Peritubular dentin (PTD) borders the tubule lumens and is surrounded by intertubular dentin (ITD). Differences in PTD and ITD composition and microstructure remain poorly understood. Here, a ($\sim 200\text{ nm}$)², 10.1 keV synchrotron x-ray beam maps x-ray fluorescence and x-ray diffraction simultaneously around tubules in 15–30 μm thick bovine and equine specimens. Increased Ca fluorescence surrounding tubule lumens confirms PTD is present, and the relative intensities in PTD and ITD correspond to carbonated apatite (cAp) volume fraction of ~ 0.8 in PTD vs. 0.65 assumed for ITD. In the PTD near the lumen edges, Zn intensity is strongly peaked, corresponding to Zn content of $\sim 0.9\text{ mg/g}$ for assumed concentration of $\sim 0.4\text{ mg/g}$ for ITD. In the equine specimen, the Zn K-edge position indicates Zn²⁺ is present, similar to bovine dentin [2], and the above edge structure is consistent with spectra from macromolecules related to biomineralization. Transmission x-ray diffraction shows only cAp, and the 00.2 diffraction peak (Miller-Bravais indices) width is constant from ITD to the lumen edge. The 00.2 cAp average preferred orientation is axisymmetric (about the tubule axis) in both bovine and equine dentin, and the axisymmetric preferred orientation continues from ITD through the PTD to the tubule lumen. These data indicate that cAp structure does not vary from PTD to ITD.

Keywords

dentin; tubule; x-ray diffraction mapping; x-ray fluorescence mapping; zinc; carbonated hydroxyapatite

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Disclosures

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1. Introduction

Dentin is a porous mineralized tissue providing toughness to the dental organ. Tubules run through dentin, extending from near the dentinoenamel junction (DEJ) to the pulp cavity. Odontoblasts produce these long open channels as they move away from the DEJ during tooth development, and the tubules (typically 1–2 μm diameter spaced 5–10 μm apart) are filled with odontoblastic processes or their remnants, i.e., fluid or soft tissue. The dentin surrounding these tubules contains two phases: intertubular dentin (ITD), which forms between the odontoblastic processes (tubules), and peritubular dentin (PTD), which grows after ITD mineralizes and around the circumference of the tubule and into its lumen. ITD makes up most of the volume of dentin and is a composite consisting of a matrix of collagen fibrils discontinuously reinforced with nanoplatelets of carbonated hydroxyapatite (cAp) making it strong but tough [26]. PTD is more highly mineralized than ITD [3, 4, 21] but contains little to no collagen [21–23] making it significantly harder and stiffer than the ITD. It remains unclear exactly how cAp in ITD and in PTD are related to each other and how PTD forms adjacent to ITD.

Earlier, diffraction and fluorescence mapping around bovine dentin tubules was reported in 1 μm thick samples [1, 2]. A 250 or 200 nm diameter beam was scanned across the specimens and mapped the variation of Ca and Zn fluorescent intensity and of the carbonated apatite (cAp) crystallographic preferred orientation around tubules. Crystallographic preferred orientation did not change between positions remote from and adjacent to the tubule. Further, these studies found Zn^{2+} concentrated around the tubule lumens but enhanced Ca signal near the tubules was not clearly observed, i.e., hypermineralization characteristic of PTD vs ITD [3–5].

The earlier studies [1, 2] had limitations. Diffracted intensities, originating from the entire sample thickness, were very weak because there were relatively few nanoplatelets irradiated at each position ($\sim 5 \times 10^3$), and noisy maps resulted. Further, data from 1 μm mineralized samples may be dominated by surface-related sample preparation artefacts. Increasing the sample thickness to 20–30 μm does not appreciably affect absorption of 10 keV x-ray photons but increases the number of platelets sampled to $\sim 1 \times 10^5$, improves counting statistics by more than a factor of four (for the same counting time) over those reported earlier [1, 2] and provides signal dominated by material farther than 1 μm from the surface.

A consequence of increasing sample thickness is that lateral “averaging” of structural details can result. For 200 nm mapping, a 1 μm thick sample possesses an aspect ratio (thickness to beam width) of 5, and a 20 μm thick sample has an aspect ratio of 100. Mapping in $\sim 20 \mu\text{m}$ thick specimens would be problematic, therefore, if closely-spaced, sharply-defined structures were present instead of structures which change gradually with lateral position and which are relatively uniform through the specimen thickness. Earlier tubule mapping ($\sim 200 \text{ nm}$ wide x-ray beam, $\sim 1 \mu\text{m}$ thick specimen) showed, however, that fluorescent and diffracted signals varied smoothly around tubules [1,2]. It should, therefore, be possible to map the surroundings of tubules running through the thicker specimens and perpendicular to the surfaces, and demonstrating this is the first goal of the studied reported below.

The earlier studies [1, 2] were also limited in the number and locations of tubules studied, in the number of teeth observed (one incisor and one molar), in the species examined (*Bos taurus*, breed black Angus) and in the age of the animal (12–18 months). The study reported below examines tubules in dentin from additional animals and an additional species (*Equus ferus*). The earlier studies also did not clearly identify enhanced levels of Ca where PTD was expected, and, given that the presence of PTD can be quite variable within bovine teeth, it is unclear whether these results reflect an intrinsic limitation of using 10 keV photons to excite Ca fluorescence (K α energy of 4.03 keV) to look for potentially small changes in Ca on top of an already large signal from ITD or whether the tubules sampled did not contain prominent PTD. Therefore, the third goal of this study is to identify and characterize PTD by mapping cAp diffracted intensity and crystallographic preferred orientation and Ca and Zn fluorescent intensity around tubules in 15–30 μ m thick bovine and equine samples.

2. Materials and Methods

2.1. Sample Preparation

Erupted bovine pre-molars were extracted from two animals 12–18 months in age and cast in LR white (Electron Microscopy Sciences, Hatfield, PA). An equine incisor from a ten year old animal was prepared in a similar way. Cuts ~125–150 μ m thick were made perpendicular to the nominal tubule axes using an Isomet 1000 wafering saw (Buehler, Lake Bluff, IL). These sections were polished by hand to thicknesses of ~15–35 μ m using 500 grit SiC polishing paper placed between two glass microscope slides; this kept the two faces of the specimen as flat as possible although slight variations in thickness from side to side were noted. Polishing ended when periodic observation with transmission optical microscopy revealed clear tubule images. Although the bovine samples were thicker than the equine samples (25–30 μ m for the former compared to 15–20 μ m for the latter), precise thickness measurements are not available. The authors noted no preferential material removal (greater than a few tens of nm) between ITD and PTD, consistent with earlier work [22, 23].

Samples examined include bovine pre-molar root and crown and equine incisor crown volumes (Table 1). The samples were wiped with lens tissue and then briefly rinsed with ethanol to remove the worst of the polishing debris. Longer rinsing or specimen submersion in liquid was specifically avoided to prevent possible alteration of the Zn near the tubule lumens. Transmission optical microscopy was used to identify areas where the tubules were perpendicular to the section and were not affected by debris.

2.2. X-ray Examination

The thick sections were examined at station 2-ID-D, APS, using the x-ray microprobe described elsewhere [6]. Each dentin section was glued with clear nail polish to a 1 mm thick Al plate with a hole cut into it. Note that the nail polish was always kept out of the incident beam. The plate was glued to a rod fitting into the goniometer. The samples were placed normal to the incident x-ray beam at the focal plane of the x-ray zone plate and at the rotation center of the diffractometer as shown elsewhere [1, 2]. The samples were examined using an x-ray beam with ~200 nm $\times$ ~200 nm cross-section and 10.1 keV energy (10^{-4} energy band width with 3×10^9 photons per sec within the area of the beam). Both x-ray

fluorescence and diffraction measurements were performed simultaneously on all of the samples.

The fluorescence intensities were mapped using a Vortex-EX silicon drift detector (SII Nanotechnology USA, Northridge, CA) positioned as close as possible to the specimen and aligned to collect x-rays emerging nearly parallel to the front surface of the specimen and in the plane of the storage ring for the best signal-to-noise ratio. Windows were set in the multichannel analyzer at energies spanning the Ca and Zn K lines. The air path was too long for detection of Na or Mg peaks.

Wide-angle x-ray scattering (diffraction) peaks from cAp were mapped with a QUAD-RO CCD detector (Princeton Instruments, Trenton, NJ). The detector was placed behind the specimen at a sample-to-detector distance of 34.2 mm normal to the transmitted beam and centered on it. At this sample-to-detector distance, the 00.2¹ and unresolved 21.1+11.2+30.0 cAp diffraction rings (20.4 and 25.0–25.9 °2 θ , respectively) were recorded in their entirety. The samples were positioned in the x-ray beam by observing radiographs of the specimen produced by the unfocused beam after moving the order sorting aperture out of the beam. The radiographic contrast was poor, but borders of the sample could be observed through their strong phase contrast. Using these edges as references for comparison with previously recorded optical transmission micrographs, each sample was translated to an area where the tubules were nearly perpendicular to the section plane.

Rapid scans over larger areas, typically 25 × 25 μm^2 , identified individual tubules for mapping (400 nm translation steps, acquisition of only fluorescent signal, 1 s/position, ~1.5 hr duration). After selecting specific tubules, high resolution scans were recorded (200 nm steps, 10–25 s/position integration for fluorescence, 15–30 s/position integration for the diffraction pattern, respectively) covering one or two tubules, spanning areas of 5 × 10 to 8.8 × 8.8 μm^2 (see Table 1 for the specifics for each sample and area scanned). Covering each area required 12–16 hr.

2.3. Fluorescence Analysis

To account for variations in x-ray beam intensity, fluorescence intensities were normalized using the incoming beam intensity measured by an ionization detector placed before the specimen. The normalized Ca and Zn fluorescence intensities were plotted as a function of position for all of the samples mapped. The tubule lumens were easily discerned in these fluorescence maps by their low Ca and Zn signals.

2.4 Diffraction Analysis

Analysis of the diffraction data was performed as described in detail elsewhere [2]. In brief, the program Fit2D [7] refined parameters including sample-to-detector distance and detector tilts. The shape of the cAp 00.2 diffraction peak was fit over 15° azimuthal bins using a pseudo-Voigt type curve after subtracting background. From these fits, the integrated intensities, measured from the area under the fitted curve, were determined for nanoplatelets

¹Note the use of Miller Bravais indices for this hexagonal crystal system.

with their *c*-axes aligned at angles of 0°, 30°, 60°, 90°, 120° and 150°±15° (angles increase in a clockwise sense). The integrated intensity of an azimuth of interest is the average of the integrated intensity of the bins on either side of the azimuth as well as the bins adjacent to the complementary azimuth (i.e., 0° ± 15° combined with 180° ± 15°). Like the fluorescence intensity values, the integrated diffracted intensities were normalized by the incident x-ray beam intensity to account for beam intensity variations. For the diffraction maps, the integrated intensities for each range of azimuthal angles were plotted as a function of position.

The radial position of the 00.2 diffraction ring, *R*, was determined from the location of the center of the pseudo-Voigt fit peak. These radial positions were then converted to *d*-spacing using Bragg's law: $\lambda = 2 d_{hkl} \sin \theta$, where λ is the x-ray wavelength, d_{hkl} is the Bragg

spacing of the *hkl* reflection and θ is the diffraction angle which is equal to $\frac{1}{2} \tan^{-1}(R/z)$ with *z* being the sample to detector distance. With the measured reference and local *d*-spacings, d_0 and d_1 , respectively, the longitudinal residual strains in the dentin were then

calculated from the definition of strain, $\varepsilon_1 = d_1 - d_0 / d_0$, and the apparent cAp Poisson's ratio measured in dentin of $\nu_{21}=0.22$ [8]. The reference position is taken at a point in the ITD far from the tubule lumens.

The full width half maximum (FWHM) of the 00.2 diffraction ring was measured as the average of the FWHM of the pseudo-Voigt fit peak for each 15° bin. From this FWHM and an assumed average longitudinal platelet size of 50 nm [9], the root mean square (RMS) strain, i.e., the distribution of cAp strain in the sampled volume, is:

$$\Delta 2\theta \cos \theta = 2\varepsilon_{rms} \sin \theta - \frac{\lambda}{t},$$

where 2θ is the FWHM of the 00.2 peak in radians, *t* is the platelet size along the *c*-axis direction, and ε_{rms} is the RMS strain.

2.5 XANES (X-ray absorption near edge structure)

X-ray absorption near edge structure (XANES) was recorded from one of the equine dentin specimens described above and a Zn metal standard using the same beam dimensions as in the mapping experiments. XANES was obtained at a position near a tubule identified as having high Zn content in earlier mapping. The monochromator was scanned over the energy range encompassing the Zn K-edge (9.630–9.730 keV in 1 eV increments), and the intensity of the Zn K α line was measured.

3. Results

Figure 1 shows transmission optical micrographs of areas of the specimens within which the maps of Fig. 2 were obtained. The tubules are more closely spaced than in the earlier studies [1, 2], and the tubules have circular cross-sections which means that they are close to perpendicular to the faces of the 15–30 μ m thick plates of dentin. The tubule diameters within the fields of view of Fig. 1 appear slightly larger than 2 μ m although, at this

magnification and through this specimen thickness, the dimensions are only approximate. The network of dark lines visible in the equine specimen arose during the final stages of sample thinning and appear to be polishing-related microcracks. There are $5\text{--}6 \times 10^4 \text{ mm}^{-2}$ tubules within the bovine sample of Fig. 1a and $\sim 4 \times 10^4 \text{ mm}^{-2}$ in the equine dentin sample of Fig. 1b.

The only diffraction peaks observed were the cAp 00.2 and unresolved cAp 21.1+11.2+30.0 peaks. Typically, detector pixels within the 00.2 ring contained ~ 450 cts, within the background near the 00.2 peak ~ 410 cts and within the 211.1+11.2+30.0 peak ~ 500 cts. The only appreciable fluorescent intensities were from Ca, Zn and P, and these intensities are given below.

Maps around one bovine and one equine tubule are shown in Fig. 2; these are typical of the other maps of tubules running perpendicular to the specimen surface (Table 1). Panel (a) shows a bovine tubule from the tooth root and (b) an equine tubule from the crown; the labels for each map appear above or below it. In each panel, the right-most columns of images map fluorescent intensity (Ca and Zn); the other maps show variation in diffraction quantities. The three left-most columns show the 00.2 integrated intensity within the specified azimuthal range. The maps adjacent to the fluorescence data show the variation of FWHM of the 00.2 peak and the total 00.2 integrated intensity ("SUM"), i.e., the sum of intensities in all of the azimuthal bins.

3.1. Bovine dentin mapping

The fluorescence maps and the 00.2 "SUM" intensity maps (i.e., integrated over the entire ring) show one tubule in the center and four other tubules near the corners (Fig. 2a). The central tubule has a diameter of 10–11 pixels (2.0–2.2 μm) horizontally and 12–13 pixels (2.4–2.6 μm) vertically. The visible portions of the other tubules appear to be similar in size and shape. There is between 1.1 and 2.4 μm (mean 1.9 μm) between the border of the central tubule and the lumen boundaries of the nearby tubules.

Within Fig. 2a, Ca fluorescence intensity varies by a factor of three, with the lowest signal being from within the tubule lumen. The highest Ca intensity arises from the lower right corner. Surrounding the central tubule, Ca signal varies slightly, by less than one-tenth the maximum within the map. The map does not show a well-defined collar of enhanced Ca that would be expected of hypercalcification and PTD. A significant fraction of the tubule lumen produces definite but weak Ca signal.

The Zn intensities in Fig. 2a range over a factor of three with the minimum occurring within the tubule lumen. In general the Zn intensity tracks that of Ca, but much more of the dentin area has intensities in the upper portion of the range. The peak Zn intensity lies adjacent to the tubule lumen, but complete rings of enhanced Zn content encircling the tubules are not clear in Fig. 2a, unlike those seen in earlier studies with much bovine thinner specimens [1, 2]. A significant fraction of the tubule lumen produces definite but weak Zn signal, consistent with the Ca map.

The maps of diffracted intensity within different azimuthal sectors of the 00.2 ring (Fig. 2a), however, show the same pattern as in the thinner specimens [2]. As the azimuthal bin rotates clockwise (to larger angle) in Fig. 2a, so does the position around the tubule that produces the maximum 00.2 diffracted intensity for that bin. Supplemental Fig. 1 explains the interpretation of the intensity variation. Within each azimuthal map, diffracted intensity varies by a factor of 15 (Table 2), and the areas of minimum intensity include both tubule lumens and ITD. Unlike the Ca and Zn maps, the integrated intensity map (“SUM”) does not show signal from within the tubule lumen. The total 00.2 diffracted intensity (“SUM”) from dentin varies somewhat around the central tubule and is weakest at the right of the central tubule in Fig. 2a. The 00.2 diffraction peak width (FWHM) is uniform except between the central tubule and the tubule in the lower right corner. Both the total integrated and FWHM do not appear to vary with distance from the edge of the tubule lumen.

The maps of Fig. 3 (Ca, Zn fluorescent intensity, 00.2 integrated intensity) are from bovine crown dentin and cover two obliquely aligned tubules located approximately 67 μm apart. In these maps, both tubules run from lower left to upper right, and, because of the inclination (relative to the surface), one cannot determine whether the tubule has a circular or elliptical lumen. The tubule width that can be measured (across the tubule) is about 8 pixels (1.6 μm) in both Fig. 3a and 3b.

Both tubules in Fig. 3 have the same orientation relative to the fluorescence detector, and this is important when considering whether variations in fluorescent intensity reflect compositional differences or other effects. In field of view in Fig. 3a, Ca intensity in the ITD is higher in the lower right (below the tubule) than in the upper right (to the left of the tubule), and in Fig. 3b it is also higher on the lower right (to the right of the tubule) than in the upper portion (above the tubule) of the map. In both cases the variation is not large: for Fig. 3a the difference is $\sim 2.9 \times 10^5$ vs $\sim 2.6 \times 10^5$ cts, and for Fig. 3b $\sim 2.6 \times 10^5$ vs $\sim 2.2 \times 10^5$ cts. The Ca intensity peaks near the lumen for both tubules shown in Fig. 3 and, although the intensity is not constant around the perimeter, it rises significantly above the signal from adjacent volumes of ITD.

For the oblique tubules in Fig. 3, increased Zn signal rings the lumens and tracks that of Ca. In Fig. 3a, Zn fluorescence is concentrated in the dentin along the tubule lumen’s major (elliptical) axis, at least in the portion of the tubule that is mapped. In the dentin near the lumen’s longer elliptical surfaces (i.e., at positions 90° around the lumen perimeter from that noted in the previous sentence), Zn levels rise slightly above those within the adjacent ITD. In Fig. 3b, the enhanced Zn signal completely encircles the tubule, and the Zn intensity varies much less around the perimeter of the lumen than it does in Fig. 3a. The upper side of tubule in Fig. 3b has $1.6\text{--}1.7 \times 10^3$ cts adjacent to the tubule and 1.2×10^3 cts in the nearby ITD; these quantities are $1.8\text{--}1.9 \times 10^3$ vs $1.5\text{--}1.7 \times 10^3$ cts, respectively, on the other surface of the tubule.

In Fig. 3, the maps of integrated intensity of the 00.2 reflection are inhomogeneous compared to the similar maps in Fig. 2. Instead of uniform intensity around the tubule and up to its lumen, the oblique tubules have very low intensity along the projection of the tubule axis. Maps of integrated 21.1+11.2+30.0 intensity (not shown) are uniform over the

entire fields of view, including the positions of the tubule lumen seen in the Zn and Ca fluorescence maps. Therefore, the inhomogeneous distribution of 00.2 intensity reflects the intrinsic crystallographic texture of the dentin surrounding the inclined tubules, as described below in the Discussion.

Transition from fully mineralized dentin to the lumen is more gradual than was seen earlier in thinner specimens [1, 2]. In Fig. 2a, Ca fluorescence and of 00.2 integrated intensity fall together going from dentin into the tubule lumen. In Fig. 3, the complication of tubule inclination and texture (mentioned in the previous paragraph) prevents comparison except across the lumen's major (elliptical) surface; here, the Ca and 00.2 integrated intensity decrease simultaneously on going from dentin into the lumen.

3.2. Equine dentin mapping

The fluorescence maps and total integrated 00.2 intensity maps ("SUM") show one tubule in the center and five other tubules around the perimeter of the map (Fig. 2b). The Zn fluorescence data show this particularly clearly. The central tubule diameter is 9–10 pixels (1.8–2 μm) in both the Ca and 00.2 maps. There is between 2.8 and 3.8 μm (mean 3.2 μm) between the border of the central tubule and the lumens of the nearby tubules.

The Ca fluorescent intensity within the center of the tubule lumen is roughly one-third of the intensity from dentin, and this map shows no evidence of material within the lumen. The peak Ca intensity near the tubule is $2.2\text{--}2.3 \times 10^5$ cts and drops to $1.8\text{--}1.9 \times 10^5$ cts in the adjacent ITD 7 or 8 pixels away. The Ca intensity varies within the ITD, being higher near the bottom of the map in Fig. 2b and lower near the top. Nonetheless it is possible to see a ring around the tubule with Ca intensity significantly greater than that of ITD. This ring is about 5 pixels wide ($\sim 1 \mu\text{m}$, about the size of the lumen radius) and does not exhibit a sharp boundary with the surrounding ITD. The authors identify this hypercalcified region as PTD.

The ring of Zn appears fairly uniform around all of the tubules in Fig. 2b and around all of the tubules examined in this equine incisor. The band of increased Zn signal is 2–3 pixels (400–600 nm) wide, is not sharply bounded and completely encircles each tubule at approximately the inner edge of the hypercalcified zone. The peak Zn intensity is 8.5×10^3 cts, Zn intensity differs by $\sim 2 \times 10^3$ cts between near tubule and ITD volumes, and there are $\sim 3.1 \times 10^3$ cts within the lumen. If the lumen intensity represents background, then the Zn signal is 2.0–2.5 times greater near the lumen than in the ITD a few micrometers away.

The 00.2 preferred orientation maps in Fig. 2b are much noisier than those in Fig. 2a. Nonetheless, the same pattern of diffracted intensity is seen as in Fig. 2a: As the azimuthal bin rotates clockwise (to larger angle) in Fig. 2a, so does the position around the tubule that produces the maximum 00.2 diffracted intensity for that bin. The tubule lumen is not seen particularly clearly in the total integrated intensity (SUM) map, consistent polishing debris collecting in the lumen and with the higher levels of background and noisier 00.2 maps. The values of the 00.2 FWHM do not vary much over the area.

3.3 XANES

Figure 4 shows XANES spectra from a position near an equine tubule and from a Zn metal foil. The maximum and minimum signals for the equine dentin were 1.4×10^3 and 1.3×10^2 cts, respectively. For the metal reference they were 2.2×10^4 and 1.7×10^2 , respectively. The position of the edge was shifted between dentin and metal, consistent with the earlier results [2], and showed Zn^{2+} was present in dentin. Intensity above the edge rose steadily for the metal specimen and first decreased and then increased for the equine dentin, with the increase beginning at about 9.705 keV. There was a small peak in the dentin spectrum between 9.685 and 9.690 keV.

4. Discussion

In the optical micrographs of the samples mapped in Fig. 2, the number densities of tubules were $5\text{--}6 \times 10^4 \text{ mm}^{-2}$ (Fig. 1a, bovine) and $\sim 4 \times 10^4 \text{ mm}^{-2}$ (Fig. 1b, equine). In the bovine dentin mapped earlier, there were 4.3×10^4 tubules mm^{-2} [1]. For comparison, Schilke and coworkers [10] measured $2.1\text{--}4.7 \times 10^4$ tubules mm^{-2} within bovine dentin, and a micrograph of equine incisor dentin of Muylle et al. [11] showed 3.1×10^4 tubules mm^{-2} . The spacings between tubules derived from the diffraction and fluorescence maps are also smaller in the samples in the present study than those in the earlier studies and the tubule diameters are a bit larger [1, 2]. These differences are not particularly unusual for bovine or equine dentin, and the samples studied here are, therefore, representative of the tissues.

Within the tubule lumen, significant Ca and Zn fluorescent intensity appeared in Fig. 2a but not in Fig. 2b. This could be remnants of the odontoblast process but is more likely polishing debris because such signal was not seen in microtome-produced $1 \mu\text{m}$ thick specimens [1, 2]. One would expect polishing debris, however, in both samples, and it may be that the debris in the equine sample may lie far enough below the specimen surface that signal cannot reach the fluorescence detector. The 00.2 diffraction data support the supposition of some debris within the equine tubule lumens: the 00.2 integrated intensity map of Fig. 2b, unlike that in Fig. 2a, shows points of significant diffracted intensity within the tubule lumen as defined by the Ca and Zn maps.

4.1 Estimates of Ca and Zn content

The Ca signal near and away from equine tubule differed by 4×10^4 cts; for reference, the nearby ITD signal was $1.8\text{--}1.9 \times 10^5$ cts. If the weight fraction of cAp mineral within the ITD was ~ 0.65 and all of the Ca was associated with cAp, then the difference in fluorescent intensities suggests that the cAp weight fraction within the hypercalcified region is ~ 0.8 . In human teeth studied with electron-excited x-ray spectroscopy, one study found that the Ca $\text{K}\alpha$ intensity from PTD was 70% of that of pure hydroxyapatite vs 60% in ITD [12]; another that PTD contained 40% more Ca than ITD [13] and a third that up to 10 wt % more Ca was present in PTD than in ITD [14].

The Zn signal in equine dentin (Fig. 2b) is 2–2.5 times greater near the tubule than away from it, and this magnitude of difference is seen in bovine dentin as well. The authors know of no data on Zn content in bovine or equine dentin, but equine dentin probably contains Zn levels comparable to human dentin ($\sim 0.4 \text{ mg/g}$ of Zn [15, 16]; [33] gives a lower Zn content

for bovine and porcine dentin compared to human). Taking this to be ITD's mean Zn composition (i.e., for purposes of this estimate, assuming the high concentrations near the tubule balance the Zn deficient volumes within the lumens), the Zn composition near the tubules peaks at ~0.9 mg/g. Similar near-tubule compositions are seen in bovine dentin. With these low concentrations, it is not surprising that the authors could not detect Zn concentration near tubules using electron-excited energy dispersive x-ray spectroscopy and integration times extending to 5 min per point.

Several observations indicate that the Zn concentration is not from an extrinsic source (e.g., the polishing process or contamination with the material in which teeth were cast or the material used to affix the sample to the aluminum holder). Earlier mapping with samples prepared by microtome sectioning (1 μm thickness) revealed the same pattern of Zn around the tubule lumens [1, 2]. Similar concentrations of Zn were noted at mineralization interfaces in bone [34]. Even if the casting material (LR White) or nail polish (used to glue the samples to the Al holder) somehow found their way to tubules examined, they cannot be the source of the Zn signal because we observe only background levels of Zn when we purposely place the beam on these two materials.

4.2 Peritubular dentin

In the Ca fluorescence maps of Fig. 2b, the separation between the border of the central tubule and the lumens of the nearby tubules varies between 2.8 and 3.8 μm (mean 3.2 μm). The observed ring of high Ca content extends about 5 pixels (~1 μm) from the lumens, and thus there are roughly equal fractions of the map containing "high" Ca and "low" Ca, consistent with reports that equine dentin contains roughly equal volume fractions of ITD and PTD [17]. Sharp boundaries between ITD and PTD are not observed, unlike in earlier studies with back scattered electrons [5] or with soft x-rays [3, 4], but this is expected given the through thickness averaging described in the Introduction. Likewise, the lumen edges did not appear as sharp as in 1 μm thick specimens [1, 2].

Bovine tubule lumen diameters were the same for Ca fluorescence and for the cAp 00.2 integrated intensity, and these signals were proportional from the ITD through the PTD. In the equine specimens, the tubule diameters were also equal for the two modalities; Ca rose near the tubules but the maps of 00.2 integrated intensity were too noisy to determine whether a similar increase was present. Within the sensitivity of the experiments, therefore, Ca is associated only with crystalline cAp.

Hypercalcified zones were observed around both bovine and equine tubules using 10.1 keV incident x-radiation although identifying PTD through Ca fluorescence was somewhat more challenging than expected *a priori*. Microradiographs in the literature [3, 4] showed PTD quite clearly using soft x-rays (5 kV tube potential) so that the spectrum of x-rays spanned that of the Ca K-absorption edge [18] and did not extend much above it. As a result, the soft x-ray imaging was extremely sensitive to small changes in Ca content, much more so than the present experiments. Mapping with incident energies closer to the Ca absorption edge should, in future mapping, improve differentiation of ITD and PTD. Nonetheless, the current mapping experiments demonstrate contrast sensitivity sufficient to differentiate PTD from the surrounding ITD.

4.3 Mineral organization in dentin

Diffraction shows the only crystalline material is cAp and cannot rule out the presence of amorphous calcium phosphate. Further, diffraction plus Ca fluorescence maps demonstrate that the cAp mineral organization does not change from ITD to PTD: the preferred orientation maps do not change in going from ITD to the tubule lumen and 00.2 FWHM remains constant throughout (Fig. 2 and 3; other data not shown, Table 1; [1, 2]). This agrees with conclusion of transmission electron microscopy (TEM) of crushed dentin fractionated via density gradient methods [21]. Little collagen is reported in PTD [22, 23], although [24] disagrees, and this suggests cAp orientations (typical of ITD and its collagen fibrils) are transferred somehow to the cAp within the PTD. Because cAp 00.2 FWHM is equal for both PTD and ITD, the nanoplatelets are not lengthening along their *c*-axes to fill the PTD from the neighboring collagen-related ITD.

4.4 Presence of Zn²⁺

XANES indicated that Zn²⁺ and not metallic Zn was present around equine tubule, in agreement with earlier results from near a bovine tubule [2]. In both equine and bovine dentin, the Zn fluorescent intensity drops from the energy of the edge to 9.70 keV, the energy at which the bovine spectrum ended. This is quite unlike the metal sample where intensity rises with increasing energy. The bovine dentin spectrum may also have had a small peak in Zn fluorescent intensity at ~9.69 keV that roughly matches the position of the small peak noted in Fig. 4. In the alkaline phosphatase superfamily [19], features in the Zn spectrum similar to those in equine dentin are observed (small peak and fall then rise of intensity above the absorption edge). The XANES spectra for Zn²⁺ incorporated into inorganic hydroxyapatite cover a much smaller energy range [20] and offer no guidance as to whether the Zn is in dentin's cAp or organic constituents [20]. On balance, it appears that Zn is intrinsic to dentin and the authors suspect dentin's Zn²⁺ is associated with non-mineral components of dentin, perhaps through alkaline phosphatase, matrix metalloproteinases (MMP-2, -8, -9 and -20 in dentin [27–29] and MMP-13 in tooth pulp [30]) or factors such as osterix [31, 32], all of which are known to be present in dentin. The fact that there is a peak of Zn intensity near the tubules, i.e., a Zn signal overlaid on top of a significant uniform Zn content, also suggests that the Zn related to the mineralization front is transient.

4.5 Diffraction and preferred orientation

The equine preferred orientation maps are noisier than those of the bovine sample (Fig. 2). Two contributions are: integration time for the former is 20 s vs 30 s for the latter and the equine sample was thinner than the bovine sample and had fewer nanoplatelets of cAp contributing to the diffraction rings.

The shape of the lumen areas in the Ca and Zn maps of Fig. 3 indicate the tubules are inclined ~30° from perpendicular to the sample surface; this is consistent with numerous tubules with projected lengths ~15 µm in optical micrographs of these areas. The 00.2 integrated intensity maps for the oblique bovine tubules in Fig. 3, showed low intensities along the direction of the projected tubule axes but neither the Ca fluorescence nor the 21.1+11.2+30.0 integrated intensities (described in Results but not shown) were low.

The 21.1+11.2+30.0 integrated intensity maps demonstrate that crystalline cAp is present all around the inclined tubules, and the uniformity of these maps is not surprising, even in the presence of significant cAp *c*-axis preferred orientation. Each of these *hkl* make a specific angle with [00.1] in each nanoplatelet, the nanoplatelets' largest faces are tangent to the fibril axis and 21.1, 11.2 and 30.0, therefore, take a very wide range of orientations within any particular sub-volume of the specimen. The wide range of orientations for each reflection and the presence of three unresolved reflections combine to produce maps of very uniform intensity.

For inclined tubules, the low intensity regions in 00.2 integrated intensity maps reflect a significant local difference in the number of cAp nanoplatelets oriented for diffraction. In dentin, the collagen fibrils axes are tangential to the tubule axis [5] and the cAp *c*-axes parallel the fibril axes [25]. On the average, this arrangement of cAp is described as 00.2 axisymmetric preferred orientation (about the tubule axis), first described in [1, 2], but, at any position near a tubule, fibril and cAp *c*-axes take a limited range of orientations. The magnitude of this range depends on volume sampled. In tubules oriented perpendicular to the specimen plane, significant 00.2 diffracted intensity is observed for all positions, and this dictates, given the Bragg angle of 10.2°, that the sample's in-plane and out-of-plane orientation ranges must be at least $\pm 10^\circ$ within each sampled volume. Tilt of the tubule axis to $\sim 30^\circ$ from perpendicular to the sample plane (as in Fig. 3) rotates the *c*-axes in some volumes beyond the Bragg angle, and these volumes do not contribute 00.2 intensity. In Fig. 3, the volumes without 00.2 intensity lie along the projection of the tubule axis, and this geometry suggests collagen fibril (cAp *c*-axis) orientations deviate by less than $\sim 30^\circ$ from the plane perpendicular to the tubule axis. Positions away from the projected tubule axis could still diffract due to the range of orientations within the plane perpendicular to the tubule axis. This hypothesis (i.e., the specific range of fibril orientations) can be tested in the future by mapping the same tubule with several incident beam directions.

4.6 Nonuniform Ca intensity across maps

The Ca fluorescent intensities from the ITD vary somewhat across the maps, probably due to slightly bent specimens or to slowly-varying surface non-planarity. The result is that some sample areas are more favorably oriented relative to the fluorescence detector. With the detector viewing the sample nearly parallel to its surface, these changes in orientation need not be large. This complication is not present in the typical fluorescence mapping configuration with the incident and exit beam making angles of 45° with the specimen surface instead of 90° and an angle of a few degrees, respectively. The present geometry, however, preserves spatial resolution, a key in mapping micrometer wide features with a ~ 200 nm wide beam.

4.7 Improvements to methodology

The fluorescent intensity based estimates of Ca and of Zn compositions can be improved considerably using appropriate standards. Placing the fluorescence detector at 45° (instead of nearly parallel to the surface) may improve the counting statistics but this will require modification of the 2-ID-D scatter shielding or movement of the diffraction detector further from the specimen. Collecting high sensitivity Zn XANES and XAFS of the Zn-rich

volumes of PTD, of the lower Zn volumes of ITD, of Zn alloyed hydroxyapatite and of macromolecules (alkaline phosphatase, osteonin, etc.) important in biomineralization will also be very valuable.

Both PTD [3, 4] and ITD are quite inhomogeneous, and PTD can develop during the earliest stages of predentin mineralization or much later [5]. Further replicates should be collected to insure that the Ca and Zn concentrations are representative.

The pattern of 00.2 cAp diffracted intensity around inclined tubules suggests that the majority of collagen fibrils have axes no more than 30° from the plane perpendicular to the tubule. This inference could be confirmed directly using a thick specimen containing tubules running perpendicular to the surface and recording diffraction maps first with the beam parallel to the tubule axes and again with the sample tilted (e.g. angles of 15° and 30°): the pattern of 00.2 intensity would change but that of 21.1+11.2+30.0 would not.

4.8 Conclusions and future directions

The present study uses specimens substantially thicker than those used before [1, 2], and this does not appear to affect accuracy but greatly increases diffracted intensity and improves sample handling. With thicker specimens, most of the diffracting volume is well away from the polished surfaces and is unaffected by sample preparation. The authors are quite fortunate to obtain 3–4 days of beam time for the mapping experiments each of three scheduling cycles annually, and this, coupled with the 12–16 hr required for diffraction mapping of a single tubule and the surrounding ITD, means that the number of samples which can be studied are quite limited. Mapping with a (200 nm)² x-ray beam, therefore, will never replace SEM or TEM for many applications, but x-ray excitation of fluorescence is orders of magnitude superior to that of electron microprobes, and diffraction mapping of nanocrystalline solids is impractical with electron-based techniques unless one goes to extreme sample thinning required for TEM.

There are a number of interesting conclusions. The organization and crystal size of cAp does not change on going from ITD through PTD to the tubule lumen; these suggest that similar cAp patterning mechanisms are operating, despite the dearth of collagen in PTD. Data on inclined tubules are consistent with the majority of ITD collagen fibrils having axes no more than 30° from the plane perpendicular to the tubule. If ITD contains 0.65 weight fraction cAp, then PTD's weight fraction is 0.8. The peak Zn concentration appears within the PTD, and, if ITD contains 0.4 mg/g of Zn, then the peak Zn concentration corresponds to ~ 0.9 mg/g. The present study identifies Zn²⁺ in the PTD bordering the tubule lumen, and the fluorescence spectra above the Zn absorption edge resembles that reported for various organic macromolecules, consistent with the hypothesis that Zn is involved in mineralization in newly forming PTD.

Based on the enhanced levels of Zn around both equine and bovine tubules, we hypothesize that similar concentration will be seen around tubules in other vertebrate species. We also speculate that the Zn is active in mineralization and is a marker of ongoing processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Optical micrographs of bovine (a) and equine (b) thin sections. The black circular features are individual tubules, and the maps shown in Fig. 2 were from these areas.

Figure 2.

Crystallographic preferred orientation maps from the 00.2 diffraction patterns (left three columns); integrated intensity and 00.2 peak full-width half-maximum maps (labeled “SUM” and “FWHM”, respectively, fourth column from the left) and fluorescent intensity maps (right-most column). (a) Bovine tubule from premolar root of animal 1. The maps cover $7\ \mu\text{m} \times 7\ \mu\text{m}$. (b) Equine tubule from within the incisor crown. The maps cover $8.2\ \mu\text{m} \times 8.2\ \mu\text{m}$. The text above each map identifies what quantity is being mapped: i.e., the 30° map shows the 00.2 integrated intensity from the azimuthal range $30 \pm 15^\circ$ added to that of $210 \pm 15^\circ$, and “Ca” and “Zn” label maps of these fluorescent intensities. In each map, the color range is linear and scaled to the maximum and minimum values in that map. The color bar on the left applies to the diffraction-derived data) and that on the right to fluorescence data.

Figure 3.

Maps of Ca and Zn fluorescent intensities and 00.2 integrated intensity for two inclined tubules from bovine specimen 2. The map in (a) covers 7.4 μm × 7.4 μm and that in (b) 6.0 μm × 6.0 μm. The labels are as in Fig. 2 except placed in the upper corner of each map. The linear color bar appears at the left and scaling is to the maximum and minimum in each map.

Figure 4.
XANES spectra from a position near an equine tubule and from a Zn metal foil. The fluorescence counts from the metal foil were divided by a factor of 10 to put both plots on approximately the same scale.

Table 1

Experimental parameters for maps around bovine (12–18 month old black Angus) and equine (10 yr old mixed breed mare) dentin tubules.

Animal	Scan	R/C*	area μm × μm	count time		position (μm)	** Fig. N or #
				diff	fluor (s)		
Bovine 1	34	R	5 × 10	30	25	—	N
	43	R	7 × 7	30	25	631	2a
Bovine 2	48	C	7.4 × 7.4	30	23	—	3a
	78	C	6 × 6	15	11	67	3b
Equine	12	C	7 × 7	20	10	—	N
	19	C	8.2 × 8.2	20	10	3	2b
	48	C	8 × 8	20	15	—	N
	54	C	8.8 × 8.8	20	15	****	N

Notes:

* Section from tooth root (R) or crown (C).

** Separation of second scan from first.

*** Whether a map is not shown (N) or the figure number (#). **** Separation not recorded but > 100 μm. Diffraction detector = diff; fluorescence detector = fluor.

Table 2

Maximum and minimum intensities for the panels of Fig. 2 and 3 (2a is bovine root dentin, 2b is equine crown dentin and 3a, b are bovine crown dentin). Intensities in the preferred orientation maps (0, ..., 150, sum) are fractions of incident beam intensity; the full-width half-maximum (FWHM) values are for 00.2 and are in rad and the Ca and Zn fluorescent intensities are in cts. The row of dots indicate values that are the same as in the left-hand column.

Fig. 2a	0	30	60	90	120	150	Sum	FWHM	Ca	Zn
max	4.5×10^{-3}						0.017	4.5×10^{-5}	2.9×10^5	1.8×10^4
min	3×10^{-4}						0.003	3.1×10^{-5}	9.3×10^4	5.7×10^3
Fig. 2b										
max	5×10^{-3}	5×10^{-3}	7×10^{-3}	5×10^{-3}	5.3×10^{-3}	5×10^{-3}	0.03	6×10^{-6}	2.5×10^5	8.4×10^3
min	0	0	0	0	0	0	0	2.5×10^{-6}	8.3×10^4	3.1×10^3
Fig. 3a										
max	-	-	-	-	-	-	0.012	-	3.2×10^5	3.0×10^3
min	-	-	-	-	-	-	0.006	-	2.2×10^5	1.2×10^3
Fig. 3b										
max	-	-	-	-	-	-	0.01	-	2.9×10^5	2.1×10^3
min	-	-	-	-	-	-	0.003	-	1.5×10^5	8.7×10^2