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MICROPROBE ANALYSES OF THE POTASSIUM-CALCIUM DISTRIBUTION RELATIONSHIP IN PREDENTINE

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Abstract

Apex regions of continuously growing incisors of Wistar rats were quickly dissected, shock-frozen in liquid nitrogen-cooled propane, freeze-dried at -80°C and infiltrated with Spurr's resin. 400nm thick dry sections were cut with a diamond knife on an ultramicrotome. Relatively flat sections were transferred with an eye lash onto collodium coated aluminum grids. They were flattened with a glass stick and by placing another collodium coated aluminum grid just on top of the first one, exerting a uniform pressure. After carbon coating the sections were observed using the backscattered and secondary electron signals in a scanning microscope. The predentine was analyzed for calcium and potassium with an energy dispersive x-ray analysis system. The x-ray spectra revealed in the predentine regions with beginning dentine formation, near the apex, an uneven K-distribution with very low as well as more prominent x-ray peaks. The K peaks were always lower than those of calcium. In areas with advanced dentine formation, prominent K-peaks were always observed. They were normally higher than the Ca-peaks up to a distance of 5-10 μm from the dentine border. Closer to the dentine border the K concentration decreased while the Ca-peak increased. This might indicate that (besides Na) K is used to balance the negative charges of the macromolecules till K is replaced by Ca at the onset of apatite crystal formation.

KEY WORDS: Microprobe analysis, predentine, calcium, potassium

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Introduction
(in special honour to Dr. T.A. Hall)

Hard tissue formation takes place in several steps leading from a precursor soft tissue to a mineralized tissue. It is necessary to clarify the sequence of events of this multistep process in order to obtain a deeper knowledge of physiological biomineralization. One important step is the transport and extracellular accumulation of calcium and phosphate, the main constituents of the developing mineral, which can be well analyzed by microprobe analysis. In order to learn the application of quantitative electron probe x-ray microanalysis I went to Dr. T.A. Hall in the EM-Unit of Dr. V.E. Cosslett in the Cavendish Institute in Cambridge in March 1966.

Since I was probably the first foreign guest to learn the quantitative continuum method (the Hall method) in biomedical research a few personal remarks might be allowed in this paper dedicated to Dr. Ted Hall.

When I came to Dr. Hall's group he had just evaluated and started to apply a first equation for quantitative element analysis. By intuition and because of a deep knowledge of the theory and experimental results of x-ray physics he had come to the conclusion that a direct proportionality exists between x-ray counts in an energy band of the x-ray continuum and the mass analyzed by the electron beam, and that this can be used to determine the elemental content per mass unit. In this first equation (Marshall and Hall 1965) it was possible to analyze only very few elements at low concentration. It was very impressive to see the intellectual energy with which Dr. Hall tried to improve his equation in the following months of my stay and in the following years of my repetitive visits for analyses up to 1970 (Hall 1971). It was not only impressive to see the constant energy with which he worked on the theory of his equations but also the practical sense with which he helped a newcomer like me to start and carry out my first measurements.

Already after some months we had got the first conclusive as well as surprising results for an unmineralized hard tissue matrix, the predentine. They were also the first or among the first quantitative results in this field. In addition to the excellent coincidence of deep

knowledge in theoretical physics and excellent experimental ability it was, above all, the great human quality of Dr. Hall, coming from deeper philosophical sources, which gave my stay and that of my wife (who assisted us in tissue preparations) not only much enrichment, but made it the most important year of my life in science.

In these first quantitative analyses we measured the Ca- and P-content in the predentine of 4-6μm cryostat sections from rat incisors. The Ca-accumulation was generally in the range of 0.2-0.6% of the dry weight (w/w) while the P accumulation was in the range of 1-2% w/w, resulting in Ca/P ratios which were sometimes only 1/10 of the apatite value (Höhling et al. 1967, 1968). It should be mentioned that these first results were obtained with an old experimental microprobe which had only one semifocussing crystal spectrometer of poor reflection quality. Though we had to apply high specimen currents these first results are in good agreement with those of later measurements (Nicholson et al. 1977). This was probably due to a thick sandwich coating of aluminum, which prevented most of the Ca and phosphate groups from escaping the sections.

After this collaboration with Dr. Hall we carried out microprobe analysis on the epiphyseal growth plates of different animals and on the matrix of turkey leg tendon (Krefting et al. 1981, 1984, Barckhaus et al. 1980, 1985). For all these hard tissue matrices microprobe analysis revealed a calcium-phosphorus concentration product which was more than 100 times higher than the CaxP ion product of about 2 $(mM)^2$ which is necessary for an in vitro mineralization of collagen rich systems. So these analyses led to the important result that high calcium and phosphate reservoirs exist in the matrix for crystal formation. During our microprobe analyses on turkey tibia tendon and above all on the longitudinal septae of the epiphyseal growth plate we have observed high extracellular concentrations of sodium and potassium. They were with 3-5% w/w much higher than the Ca content of the corresponding region. The question was raised whether the high extracellular K content in the longitudinal septae is due to tissue preparation or does really exist in vivo.

In order to study more deeply the question of a possible extracellular K accumulation we returned to predentine, the tissue with which the first author of the present publication had started microprobe work in collaboration with Dr. Hall (Höhling et al. 1967, 1968). A further reason for this study was that Larsson et al. (1988) had analyzed micropuncture fluid, aspirated with a micropipette from the predentine of rats, and found elevated K concentrations. The study reported in this paper was started during a stay on a Japanese fellowship at the Dental School of Nihon University at Matsudo. We could not yet apply the Hall method in this study but want to apply it in near future.

Materials and Methods

Young Wistar rats (weight: about 150 g) were anesthetized. The upper and lower jaws were dissected, opened and the soft apex of the continuously growing incisors were separated from the jaw bone as fast as possible and transferred to liquid nitrogen-cooled propane. Though this preparation was done by an expert the time from dissection of the jaw to transferring the apex into the propane bath took 4 minutes at minimum and 8-10 minutes at maximum. Since the odontoblasts are long prismatic cells with long processes and since the predentine zone has a width of about 25 μm we assume that an extensive ion exchange during this time from the cells into the deep predentine and vice versa has not taken place. The tissue was freeze-dried at -80°C in an Edwards-Pearse tissue-dryer for 6 days. After a slow warming up in vacuum with dried air the tissue was infiltrated with chlorine free Spurr's resin and the polymerization carried out at 60°C.

On the basis of our earlier microprobe results for Ca and P which were carried out on 4-6 μm cryostat sections and on dry thin sections of such shock-frozen, freeze-dried and embedded incisors (reviewed in: Nicholson et al. 1977) we assumed also for this project that the infiltration of the tissue with Spurr's resin would not transport appreciable amounts of calcium and potassium over distances larger than the diameter of the ice crystals being formed during freezing.

Dry sections with a thickness of 0.4 μm were cut with a diamond knife on an ultramicrotome. Relatively flat sections were obtained on the top of some rolled up sections and transferred with an eyelash on to 100 mesh aluminum (Al) grids, some of which had been Formvar-coated. The dry sections on the Al grids were further flattened by putting a second freshly coated Al grid on top of the grid with the sections and exerting a slight continuous pressure with a glass rod, under a stereomicroscope. The sections were coated with carbon.

The element analyses were carried out in a JEOL scanning electron microscope, equipped with a Si(Li)-detector system using the secondary electron image during analyses and the electron backscatter image for a better morphological documentation (Fig. 1, 3). In this project we analyzed the predentine a) near the apex, where only a very thin dentine layer had been formed, and b) in regions at greater distance from the apex, in which already a wide dentine layer had developed.

An accelerating voltage of 25 kV and an inclination angle between section plane and detector of 40° was chosen. Point analyses were carried out with a beam diameter of about 1-2 μm. The measuring time was 100 sec. Since in the program for automatic background subtraction the subtracted background did not coincide with the real background we carried out the background subtractions for K and Ca on the original x-ray curves.

Results

General remarks

Continuously growing rat incisor is an excellent model to study hard tissue formation in the different

stages of development. Dentine formation starts at the tooth apex and advances, with increasing thickening of the dentine zone, in the direction of the cusp. At a certain distance from the apex, enamel is formed on top of the dentine at the labial side and cementum at the lingual side, also advancing in the direction of the cusp. Consequently, by analysis of cross sections through the developing incisor it is possible to analyze dentine formation in its earliest stages near the apex and in advanced stages in the direction of the cusp.

Potassium and calcium analysis in early dentine formation

Fig. 1 shows a backscattered electron image of early dentine formation with a width of the dentine zone of about 13 μm. Morphological details of the predentine zone are not visible. Since the width of predentine is about 25 μm nearly all microprobe measurements were kept within this range to be sure that only predentine, not the adjacent odontoblast layer, was analyzed. About 30 point analyses in predentine at this stage of development were carried out. Nine analyzed microareas of one series of measurements are indicated in Fig. 1. These first measurements were partly carried out at a distance of about 6-8 μm from the dentine border but not in a systematic way, i.e. with gradually increasing distance from the dentine border along a line perpendicular to the dentine border (as was done in Fig. 3).

It was found that potassium (K) is not evenly distributed in predentine, and that microareas with a prominent K x-ray peak can be found close to regions with a low peak. At the dentine border the K x-ray peak was practically no more visible (Fig. 2). Fig. 2a shows the x-ray spectrum of measuring point 5 in Fig. 1, with a distance of 7 μm from the dentine border. A prominent K-K_β peak can be observed adjacent to an even higher Ca-K_α peak. (The overlapping of the K-K_β peak with the Ca-K_α peak does not cause problems of interpretation since in all previous quantitative Ca measurements we have found Ca contents generally in the range of 0.3-0.6% w/w; (Höhling et al. 1967, 1968)). The K/Ca ratio of the x-ray counts is about 0.7.

The x-ray spectrum of Fig. 2b, for measuring point 3 of Fig. 1, which has nearly the same distance from the dentine border as measuring point 5, shows a much lower K-K_α peak also a lower Ca-K_α peak. This would mean a much lower K concentration, assuming that the section thickness in these neighbouring zones is about the same. The K/Ca ratio of the x-ray counts is about 0.4, nearly half the K/Ca ratio which was found for the values of spectrum Fig. 2a. The question arises whether a microarea was analyzed which contained little predentine but parts of a hole (a remnant of an ice crystal) or which contained parts of an odontoblastic process. The K/Ca x-ray count ratios of this predentine zone are mainly in the range of 0.2 to 0.7 showing that all K x-ray peaks were lower than the Ca peaks.

Fig. 2c represents an x-ray spectrum of a measurement directly at the dentine/predentine border. The Ca-K_α peak is prominent, but a K-K_α peak is no more visible, indicating a loss of K in predentine approaching the dentine border.

Apart from the K and Ca x-ray peaks in predentine also Cl, S, P, and Si peaks were always observed besides the Al peak caused by a secondary excitation of the bars of the aluminum grid. Also mostly a Na peak was prominent, at the left side of the Al peak (Fig. 2a), which has not been denoted by "Na". It overlaps partly with the L-lines of Cu and Zn which were excited from the brass block surrounding the carbon holder for the aluminum grids. Cu-K_α and Zn-K_α peaks always appeared in the spectrum in spite of carbon painting on most parts of the brass holder.

Potassium-calcium analyses in regions with advanced dentine formation

Fig. 3 shows a backscattered electron image of an advanced stage of dentine formation with a broad dentine zone containing odontoblastic processes. About 30 measurements were carried out in 9 series, with point measurements along a line perpendicular to the dentine border, proceeding from the dentine.

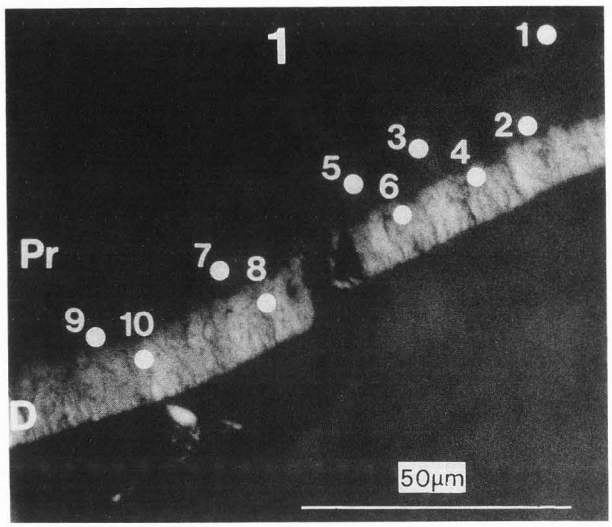
Fig. 4a represents the x-ray spectrum of measuring point 3 of series 2 in Fig. 3, with a distance of about 5 μm from the dentine border. Besides high Ca-K_α and P-K peaks prominent K-K_α is present. The high Ca and P x-ray peaks might be explained partly by tiny mineralized particles which might already appear in this region. Because of the high Ca-K_α peak the K/Ca ratio of the x-ray counts is only 0.2.

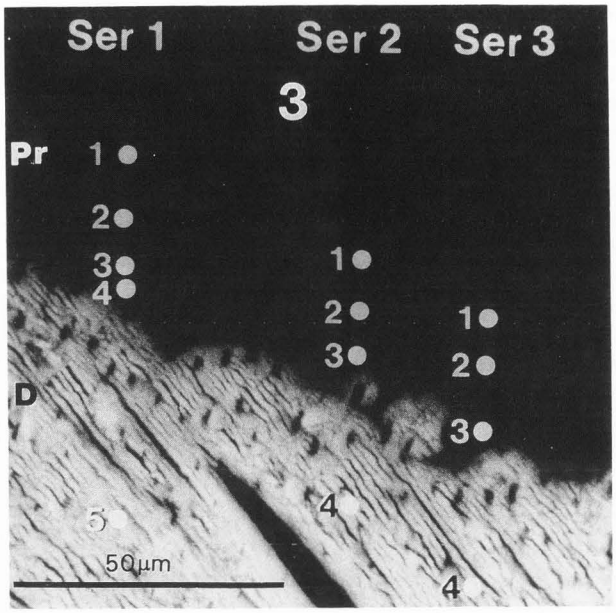
Fig. 4b shows the x-ray spectrum of the measuring point 2 of series 2 in Fig. 3, with a distance of 14 μm from the dentine border. The K-K_α x-ray peak is nearly the same as in the spectrum of Fig. 4a; but the CaK_α peak is much lower so that a K/Ca ratio of the x-ray counts of about 1.6 results. In many measurements with a distance more than about 5-10 μm from the dentine border such high ratios were found.

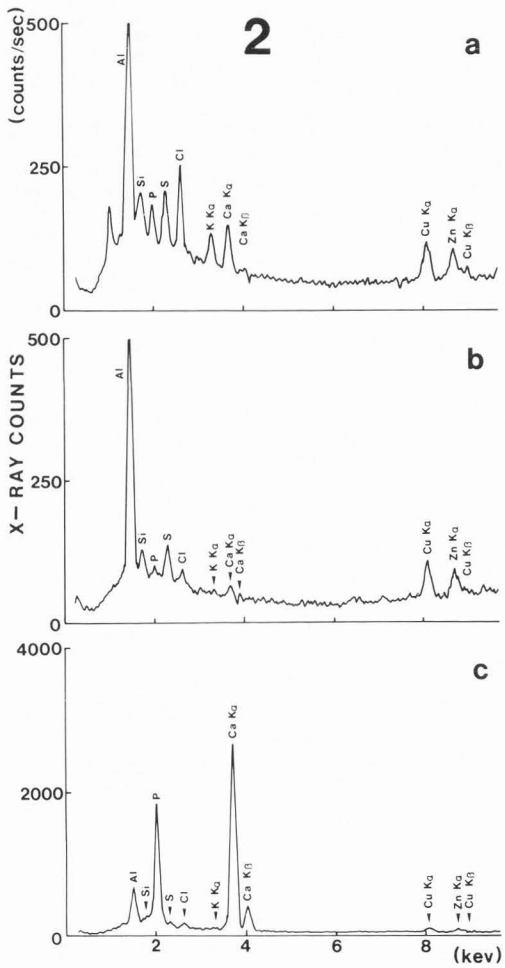
Fig. 4c represents the x-ray spectrum of measuring point 1 of series 2 on Fig. 3, with a distance of about 20 μm from the dentine border. The K-K_α and Ca-K_α peaks have nearly the same height as in the spectrum of Fig. 4b, indicating that no remarkable changes in the Ca and K concentration have taken place (assuming a nearly identical section thickness for these neighbouring regions). A K/Ca ratio of 1.7 (compared with 1.6 of the spectrum of Fig. 4b) supports this view.

Plot of the potassium and calcium from the dentine to the predentine odontoblast-border

We assumed that for a distance of at maximum 25 μm, from the dentine border to the predentine odontoblast border, the section thickness remains roughly the same. We also assume that the specimen current is relatively constant for the period of one series of measurements consisting of 4-5 points. A variability of maximally 20% is taken into account for the section thickness and the specimen current. Hence the net K-K_α and Ca-K_α x-ray counts would reflect the element content per volume. For the determination of the x-ray peaks and the







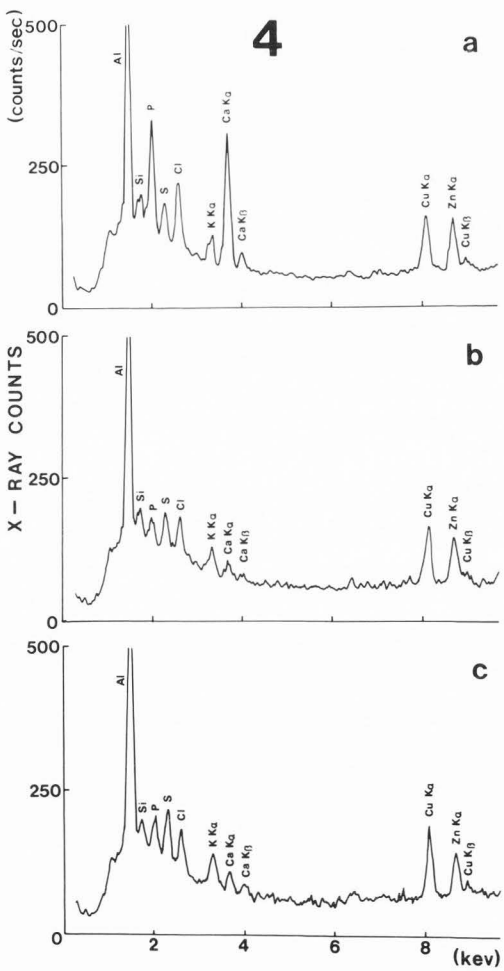


Figure 2: Energy dispersive x-ray spectra from point measurements denoted in Fig. 1.
Spectrum a: point measurement 5 in Fig. 1.
Spectrum b: point measurement 3 in Fig. 1.
Spectrum c: point measurement 4 in Fig. 1.

Figure 1: Developing dentine in rat incisor near the apex; early formation. Dry section of shock-frozen, freeze-dried, embedded tissue. Backscattered electron image. 1-9 points of measurement. D = Dentine; Pr = Predentine.

Figure 3: Backscattered electron image of developing dentine in rat incisor, advanced stage. Dry section of shock-frozen, freeze-dried, embedded tissue. Three of the series of point measurements in predentine (Pr) and dentine (D). (Ser 1 to Ser 3) are indicated.

Figure 4: Energy dispersive x-ray spectra from the point measurements of Ser 2 in Fig. 3.
Spectrum a: point measurement 3 of Ser 2.
Spectrum b: point measurement 2 of Ser 2.
Spectrum c: point measurement 1 of Ser 2.

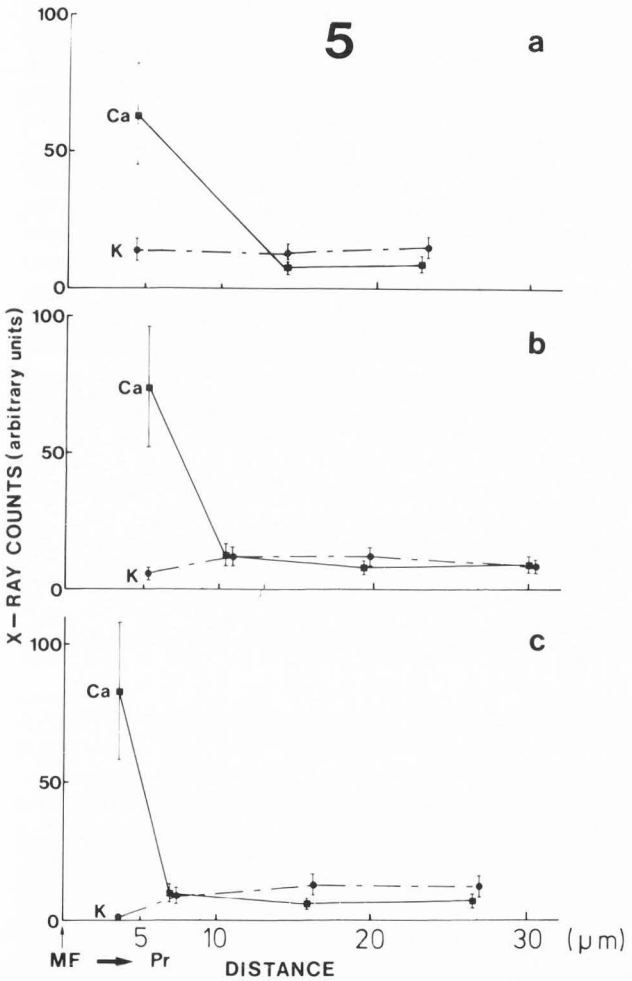


Figure 5: Plot of the K and Ca x-ray net peaks against the distance of the point measurements, partly indicated in Fig. 3. Plot a: distribution of K and Ca x-ray peaks of Ser 2, Fig. 3. Plot b: distribution of K and Ca x-ray peaks of Ser 1, Fig. 3. Plot c: distribution of K and Ca x-ray peaks of a series not indicated in Fig. 3. MF = Mineralization Front; Pr = Predentine.

subtraction of the background on the x-ray spectra (e.g., Fig. 4) an error of about 10% exists. So, for the plot of the K-K_α and Ca-K_α x-ray counts against the distance of the measurements from the dentine border a total range of error of 30% was indicated at the points of measurement in the curves of Fig. 5.

Fig. 5 shows the curves of three series of measurements, of a total of nine, partly indicated in the backscattered electron image of Fig. 3. The curves of Fig. 5a correspond to series 2 in Fig. 3, the curves of Fig. 5b to series 1 and the curves of Fig. 5c are not indicated in Fig. 3.

The K-curve of Fig. 5a shows a nearly horizontal course from a distance of about 22 μm to about 5 μm from the dentine border. This is the only curve in which a decline of K at the 5 μm distance from the dentine border could not be observed. Hence, the K content per volume is about constant. The Ca-curve shows a relatively steep rise in the range of 14 to 5 μm from the dentine border.

The K-curve of Fig. 5b also shows a relatively horizontal course from about 30 μm to 10 μm but a clear decline in the range of 10 to 5 μm, indicating a decrease in the K content. The Ca-curve shows a strong rise in the range of 10 to 5 μm.

The K-curve of Fig. 5c shows a horizontal course, as in Fig. 5a, and b, from about 26 to 6 μm from the dentine border but a clear decline in the range of 6 to 3 μm, where the Ca-curve again shows a strong rise.

In summary, the K- and Ca-curves indicate a relatively constant concentration through the main part of predentine from the predentine odontoblast border but indicate a change in the concentration near the dentine border. In the range of about 5 to 10 μm from the dentine border the K content per volume starts to decrease and at the dentine border a K-K_α peak is no more visible with the energy dispersive system. In this region of 5-10 μm (or up to 15 μm) an increase of the Ca-K_α counts was observed. For most of the point measurements with distances from the dentine border of more than 5-10 μm the K-K_α counts were higher than the Ca-K_α counts. The range of the K/Ca ratios of the x-ray counts for this region was 0.9-1.8.

Discussion

Boyde and Shapiro (1980), Hargest et al. (1985) and Krefting et al. (1981, 1984) determined high concentrations of Na and also of K, by electron probe x-ray microanalysis in the extracellular, longitudinal septum of the epiphyseal growth plate. Krefting et al. (1981, 1984) found different extracellular K contents in the different zones of the growth plate, mainly in the range of 3-6% w/w corresponding to about 150-300 mmol/kg wet weight (for a tissue water content of 80%). For these different zones of the growth plate Krefting et al. (1981, 1984) have determined a relatively constant Ca content of about 0.8% w/w corresponding to about 40 mmol/kg wet weight if one assumes a water content of 80%.

Krefting (1985, 1987) observed, by quantitative electron probe x-ray microanalysis, that an increasing intracellular Ca content is directly correlated with an increasing extracellular K content; and he concluded that the intracellular Ca and extracellular K increase are due to an ion flux because of cell damage during tissue preparation. The first author of the present paper came to the conclusion that only part of the extracellular K is due to a preparation artifact, and that a high portion of the extracellular K exists in vivo. The K ions, in addition to the dominating Na ions, would be used to balance the charges of the negative groups of the dominating proteoglycans and other macromolecules. Quint et al. (1982) found for pooled cryostat sections of the upper hypertrophic zone that about 60% of the total K was extractable with glycine buffer (pH 8), while already 70% was extractable in the zone of maximal hypertrophy, where already an intense Ca-phosphate formation had started.

Also Barckhaus et al. (1980) have obtained on dry sections of shock-frozen, freeze-dried embedded turkey tibia tendon prominent K-K_α and Na-K_α peaks in elliptic contrast-rich microareas of the unmineralized matrix near the mineralization front.

On the basis of these results and the analyses of Larsson et al. (1988) on micropuncture fluid from predentine of rats, which showed high concentrations of Na and elevated concentrations of K, we started to analyze the K distribution in predentine of rat incisors. We could not yet analyze the possibly more important Na, since the Na-K_α line overlaps with L-lines of Cu and Zn which are constituents of the brass holder. A further disadvantage is the strong absorption of the Na-K_α line in the Be window of the Si(Li) detector.

In the early stage of dentine formation microareas were found with prominent K-K_α peaks which resulted in K/Ca count ratios in the range of 0.5-0.7 (Fig. 2a). Besides these zones of K enrichment also microareas with a relatively low K-K_α peak were observed with K/Ca count ratios of only 0.1-0.3 (Fig. 2b). Probably in both regions the K content is lower than that of Ca with mean values of 0.3-0.6% w/w, corresponding to 25-50 mmol/kg wet weight (reviewed by Höhling and Fromme 1984), on the basis of 66% tissue water content for predentine, as determined by Linde (1973). Since morphological details of the predentine cannot be recognized in the scanning micrographs (Fig. 1, 3) the reason for the uneven K distribution could not be clarified. Since odontoblastic processes, which are not visible in these micrographs, probably are not yet present or not dominating in this early stage of development a possible uneven K, Ca-distribution in predentine and odontoblastic processes does not seem to be the reason. One can consider that microareas with remnants of ice crystals, which reach the μm-size, were mainly hit and caused the low K (and Ca) peak. Further, the K distribution might be more uneven in the early stage of dentine formation if it is not caused by redistribution during the infiltration with the embedding medium after freeze-drying. Consequently, more systematic analyses from the dentine border into predentine, with a better morphological representation and on thin cryosections are necessary to clarify the K-distribution.

The results which we have obtained for the zone of advanced dentine formation are quite clear (Fig. 3). All point measurements in the predentine with a distance to the dentine border higher than 5-10 μm showed prominent K-K_α peaks (Fig. 4), with K/Ca count ratios in the range of 0.9-1.8. Most of the K-K_α counts were higher than the Ca-K_α counts probably indicating a higher K content than the mean Ca content of 0.3.-0.6 % w/w (reviewed by Höhling and Fromme 1984). This would also mean that the K content is clearly higher than the 17.5 $\pm$ 6.9 mmol/l K for in vitro and the 9 $\pm$ 9.9 mmol/l for in vivo aspirations of the micropuncture fluid (Larsson et al. 1988). This would indicate a relatively high amount of bound K which could not be analyzed in the micropuncture fluid.

In the region of 5-10 μm from the dentine border the K content started to decrease and no peak was visible in the energy dispersive spectrum obtained at the dentine border. This K decrease was connected with an increase of the Ca-K_α counts in this range. This correlation seems to support our idea that K ions (in addition Na ions) are bound to balance the negative groups of the matrix macromolecules and are gradually replaced by Ca ions for the formation of the Ca-phosphate nuclei. For our preparation method we have to consider whether an appreciable K diffusion of intracellular K into the predentine has taken place during the 4-10 minutes from tissue dissection to shock-freezing or by the infiltration with the embedding medium. The characteristic decrease of K in the range of 5-10 μm from the dentine border seems to exclude a strong diffusion. It is not logical to assume that an artificial K diffusion would stop in this range before the dentine border. Nevertheless it must be tried to shorten the period of tissue dissection to freezing below 5 minutes and to carry out analyses directly on thin cryostat sections.

Concerning the increase of the Ca-K_α x-ray counts near the dentine border it must be clarified whether this is connected with a real Ca increase or whether it is due to a secondary excitation of the Ca atoms in the neighbouring dentine by electrons traversing the short distance in the section from the predentine. It is not easy to clarify this point. However, Felsmann (1987) analyzed this question of secondary excitation in a careful study on sharp gold edges embedded in Epon. He derived equations which allow a quantitative evaluation of the influence of the electron beam distance from the Au edge on the Au x-ray count rate and a simulated Au content. He also made an evaluation for the amount of secondary excitation for an edge of the calcium phosphate apatite, existing in bone and dentine. According to his evaluations the influence of secondary Ca excitation would start in the 5 μm distance range for a section thinner than 1 μm. Since we have observed a Ca-K_α count rate increase already in the distance range 5-10 μm from the dentine border, we conclude that this increase is at

least partly connected with a real increase in the Ca content. This conclusion can be correlated with autoradiographic and immunohistochemical results for phosphoproteins (e.g. Weinstock and Leblond 1973), which are dominating non-collagenous proteins in predentine. They show that these phosphoproteins, which strongly bind Ca, are preferentially secreted and deposited at the dentine / predentine border.

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Discussion with Reviewers

S.H. Ashrafi: The freezing of soft tissue with a dynamic cooling system at 80-100 psi pressure preserves tissue in an amorphous condition only up to 3-7 μm in depth. At what depth did you observe crystal formation in predentine after freezing?

Authors: We did not observe crystal formation but analyzed the elements mainly in the predentine. Since the distance from the cooling fluid to the analyzed tissue region was in the mm-range we had to accept ice crystal formation with ice crystal size in the 0.5-1 μm range.

S.H. Ashrafi: What was the reason of using 0.4 μm thick sections for x-ray microanalysis using a JEOL-scanning electron microscope?
Authors: We normally use 0.5 μm sections or thinner ones, in order to apply the Hall method for quantitative analysis, and also to avoid absorption corrections for the x-rays of most of the elements of interest.

S.H. Ashrafi: Why were K-peak heights in early predentine formation uneven? Do you think it was due to bad preservation and ice crystal formation during freezing and drying?
Authors: We do not know the reason, as was discussed in our paper. One of the 3 reasons which we discussed was really the fact that the 1-2 μm electron beam might have hit a considerable portion of such an ice crystal remnant in which only embedding material, and no matrix was present, resulting in a low K (as well as Ca) peak.

M.B. Engel: During infiltration with the Spurr resin is it possible for electrolytes and small molecules associated with lipoproteins or resin soluble components of cell processes or extracellular matrix to diffuse and shift? The values given in Tables 3a, 3b, 4a, 4b of the Hargest et al. reference actually show distinct differences between cells as well as matrix components of freeze dried versus freeze dried embedded sections.
Authors: As we have mentioned in our paper the possibility exists that by the infiltration with the embedding material part of the elements might be dislocated so that we expressed that in future also cryosections must be prepared and analyzed. Really, we have tried (but this was not mentioned in the paper) to get thin cryostat sections but failed; for this system of locally mixed unmineralized and mineralized microareas we obtained only brittle material instead of analyzable sections. We have mentioned that for the Ca-distribution, the Ca/P ratios etc. in the different zones of the predentine there did not exist drastic differences for the analyses of dry sections of shock-frozen freeze-dried embedded tissue and 0.5 μm unembedded cryostat sections. So we concluded that results of scientific value could be obtained by analysis of these dry sections provided that the results were interpreted with necessary precaution.

M.B. Engel: In view of the relatively high accelerating voltage and the relatively thin sections would one expect electron penetration without interaction with tissue elements? Since the required exciting voltage for K-K_α and Ca-K_α are close this might not affect the K/Ca ratio but more widely separated elements could have a considerable error.
Authors: We agree. We assume that the K/Ca ratio of the x-ray counts given in this paper is near the K/Ca concentration ratio.

M.B. Engel: The question of resolution is frequently troubling as the authors indicate. Approximations can be derived from theoretical calculations derived from the nomogram of Reed, S.J.B. (Electron Microprobe Analysis, 1975) and Russ J.C. (Edax Editor 6, Nr.3, 4-39, 1976).
Authors: We do not understand to which problem the comment concerning the resolution refers. For the relatively thin sections of 0.4 μm the resolution for x-ray analysis would not be much worse than 1-2 μm, the diameter of the electron probe.

M.B. Engel: Potassium is a dominant intracellular cation, neutralizing charge on intracellular negatively charged colloids. Is it likely that high K concentrations in predentine are mainly confined to odontoblastic processes with lesser distribution in the extracellular matrix? In the dentine could the K-K_α x-rays be swamped out by absorption and other interactions with the high concentrations of Ca? In some future studies the authors might consider either ZAF-based analysis or the use of standards if these modifications are available and feasible.
Authors: Important questions were raised. As communicated in our paper we regret that we could not visualize the odontoblastic processes in the instrument with the backscattered and secondary electron signals. So we have concluded that with much more probability we would have hit the dominating predentine instead of the thin odontoblastic processes with the 1-2 μm beam. Since also the gradient analyses for K and Ca were consistent we have concluded that our results were predominantly obtained from predentine. During the last week we could proceed with our analyses by the application of the transmission scanning mode in the Philips EM 301 and also visualize the odontoblastic processes. We have obtained K-K_α and Ca-K_α peaks from both structures but have not yet clarified whether the K-K_α peaks in relation to the Ca-K_α peaks are higher in the odontoblastic processes. For the analysis of K in the dentine we intend to use the more sensitive wavelength dispersive system. We can avoid absorption corrections for K-K_α and Ca-K_α for sections with a thickness below 0.5 μm.

M.B. Engel: In biologic calcification a major role has been assigned to the immobile negative charge density of the matrix. Based on theoretical considerations formulated by Willard Gibbs and a later application by Donnan and verified by experimental evidence (Engel M.B., Joseph N. R., Catchpole H.R. (1954), Homeostasis of connective tissues. Calcium-sodium equilibrium. Arch. Pathol. 68, 26-39) two general reactions seem probable: 1. The formation of complexes in which calcium is bound and 2. A distribution and concentration of calcium in ionic form which approximates the conditions of the Gibbs-Donnan equilibrium. Accordingly it can be shown that ionic calcium levels in the matrices would be higher than the concentration in blood and would vary with the square of the charge, while bound calcium would be a function of the cube of negative charge density.
Authors: We have come to the same questions from another side. First, we have treated pooled cryostat sec-

tions from the epiphyseal growth plate (Quint et al. 1982) with mild glycine buffer pH 8 and analyzed the "bound" and "unbound" elements such as Ca, K. We have also treated cryostat sections of dentine/predentine with defined amounts of aqua dest. and found that by far most of the Ca remained "bound" while most of the phosphate groups were dissolved (Nicholson et al. 1977).

J. Wroblewski: What is the advantage of mounting the sections on thin film (on the aluminum grids) and not on thick substrate (for example carbon plate)?
Authors: The first author had in mind to apply the quantitative continuum method (Hall method) which can be applied only to thin sections. During the short stay of 3 months in Japan there was no chance to apply this method which requires a good deal of effort. When we go on with our measurements we must use grids with large mesh distances in order to apply the continuum method. In this case we should use an extra collodium foil on the grids to be able to flatten the sections more thoroughly.

J. Wroblewski: Did you try to evaluate whether in your system lower accelerating voltage would not lower considerably the background radiation and improve the resolution of analysis?
Authors: No, we have not, but shall consider this point.

J. Wroblewski: Due to differences in hydration of predentine and dentine one should expect that the concentration of the embedding medium differs between two zones. How do you correct for this in the analysis of the spectra?
Authors: You are right, the concentrations of the embedding medium must differ considerably between dentine and predentine. As we have expressed in our paper, we have not compared the element contents on the basis of the mass but on that of the volume, assuming that some variations in the thickness of the section would be "smoothed" for many measurements at these short distances. In the future we have to carry out more than 4 point measurements for one series through the predentine to get more clarity about the Ca- and K-distribution in detail.