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ENERGY DISPERSIVE X-RAY MICROANALYSIS OF THE DENTIN IN RAT MOLARS AFTER CORTICOSTEROID TREATMENT

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Abstract

The aim of this study was to investigate whether the calcium (Ca) and phosphorus (P) composition of corticosteroid induced dentin was the same as in normally developed dentin. Seven rats were given corticosteroids intravenously and three rats served as controls. Energy dispersive X-ray microanalysis (EDX) was carried out on the axially sawn roots of the molars. Measurements were made at 20 sites, equally distributed in the buccal, mesial, lingual and distal direction. The results showed that the Ca/P ratio (weight %) was slightly above 2.0 in both the experimental and the control group, indicating that the corticosteroid induced dentin had a normal Ca/P ratio. However, different degrees of mineralization were found in different directions of the roots.

Key Words: Corticosteroids, dentin, calcification, electron probe microanalysis.

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Introduction

During recent years, the scanning electron microscope (SEM), equipped with an energy dispersive X-ray spectrometer (EDX) for microanalysis of mineral content, has contributed to our understanding of the normal mineral composition and mineralization of hard tissues as in bone and teeth (Boyde *et al.*, 1986; Engel, 1981; Green *et al.*, 1970; Hals *et al.*, 1988; Jean *et al.*, 1986; Kodaka *et al.*, 1991; Mellors and Solberg, 1966; Sanchez-Quevedo *et al.*, 1989). The mineral in these tissues is composed of hydroxyapatite in a crystalline form. The accuracy and precision of the EDX-method compared to other established methods of measuring mineral content, such as instrumental neutron activation analysis (INAA) and chemical analysis (ICPES), have been studied (Åkesson *et al.*, 1994) and found to be equally good for the major constituents, calcium (Ca) and phosphorus (P).

SEM/EDX-studies on pathological changes of dentin have been performed on teeth from patients with hypophosphatemic vitamin D-resistant rickets (Daley *et al.*, 1990; Hietala and Larmas, 1991) and dentin dysplasia (Jasmin and Clergeau-Guerithault, 1984; Melnick *et al.*, 1980). Changes of the dentin due to chronic renal failure, parathyroid hormone stimulation and vitamin D and calcium deficiency have been ultrastructurally investigated in human material and experimentally in rats (Clark and Wysocki, 1988; Engström, 1980; Turnbull *et al.*, 1983). In these studies, predominantly an increase of the odontoblast activity could be seen. Thus, systemic disorders may induce changes of the dentin due to alterations of the homeostatic regulation of Ca and P metabolism.

In a histologic and radiographic study of teeth from deceased patients who had received a renal transplant, excessive formation of dentin was found in pulp chambers. All patients had received corticosteroids in high doses postoperatively to prevent rejection of the renal transplant (Näsström *et al.*, 1985, 1993). An experimental study on rats showed that corticosteroids in high doses induced dentin formation in the root canals of the

molars (Näsström *et al.*, 1989). Corticosteroid induced dentin may be involved during dental treatment and, therefore, it is of interest to investigate the quality of this dentin. The aim of this study was to investigate the content of Ca and P in corticosteroid induced dentin in rat molars in comparison to normal dentin.

Material and Methods

Animals

Ten rats from an isogenous strain, Brown-Norway (BN) (ALAB, Uppsala, Sweden), were used. The rats were 3-4 months old; the male rats (n = 7) weighed 215-306 g while the female rats (n = 3) weighed 166-187 g at the start of the experiment.

Experimental and control groups

Rats of both sexes were divided into two groups, an experimental (n = 7; 5 male, 2 female) and a control group (n = 3; 2 male, 1 female). In the experimental group, the rats were given methylprednisolone (Solu-Medrone®, The Upjohn Co., Kalamazoo, MI, USA), 10 mg/kg body weight twice a day for 38 days. The three control rats were given physiological saline with the same volumes as the steroids twice a day.

Drug administration

The model of drug administration used was described by Konrad and Husberg (1979). All drugs were given intravenously through a polyethylene catheter introduced into the inferior vena cava through the right femoral vein. The catheters ended distally in a modified venflon (Viggo, Windlesham, U.K.) placed subcutaneously between the scapulae. A rubber membrane-equipped small cylinder protruded through the skin. The catheter was rinsed with physiological saline after each drug administration.

Fixation and specimen preparation

The animals were anesthetized with a 36 mg/ml solution of chloral hydrate at a dose of 1 ml per 100 g body weight given intraperitoneally. Perfusion fixation with 2.5% glutaraldehyde was performed in accordance with the model described by Heide (1973).

The maxillae were dissected out, and a block of three molars from each side of the rat was separated from the incisors and the zygomatic bone and placed in glutaraldehyde. The undecalcified block of molars were prepared according to a method described by Donath (1987) before embedding in methylmethacrylate. The left block of molars was sawn axially just below the furcation; all blocks were sawn at the same level after installing the saw at a level indicated by measurement on a radiograph of the block. The root section of the block was ground to a 300 μm thick section.

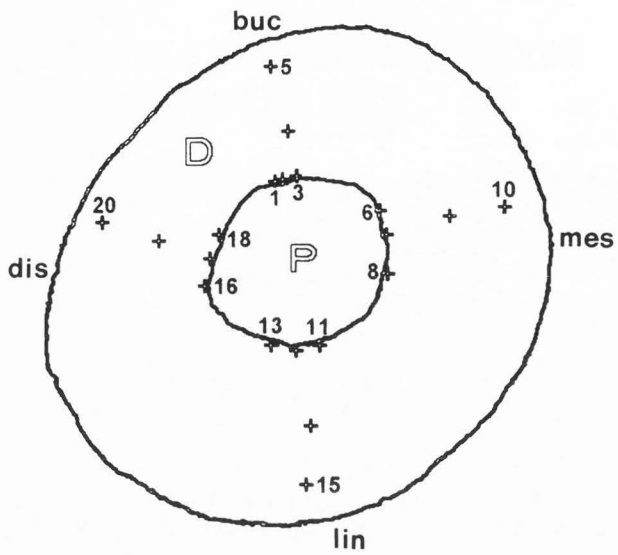


Figure 1. Schematic drawing of a root section from the second molar of a control rat. All 20 points of analysis are indicated. Close to the pulp, points 1-3 are in the buccal direction (buc), points 6-8 in the mesial direction (mes), points 11-13 in the lingual (lin), and points 16-18 in the distal (dis) direction. D: dentin; P: pulp.

The sections were examined using a Philips 515 scanning electron microscope (SEM), equipped with a QX 2000 LINK (Oxford Instrum., High Wycombe, U.K.) energy dispersive X-ray (EDX) spectrometer with beryllium window and a peak-to-background ratioing program, LINK ZAF/PB (Z = atomic number; A = absorption correction; F = secondary X-ray fluorescence; PB = peak to background) (Statham, 1979). Prior to EDX-microanalysis, all sections were mounted with carbon glue on specimen aluminum stubs. The whole surface of the stub was carefully covered with carbon glue before they were carbon coated in a vacuum evaporator. As a gain calibration element, a copper standard was mounted on the stub along with each specimen. Matrix for all specimens was methylmethacrylate.

Reference profiles for Ca and P were prepared from commercially available standards of wollastonite ($CaSiO_3$) and gallium phosphate (GaP) (Microanalysis Consultants, MAC, Agar Scientific Ltd., Stansted, Essex, U.K.). A hard, pressed tablet was made from pure calcium phosphate powder, with a Ca concentration of 39.89% and P concentration of 18.50%, and was used as a concentration standard. This standard was used at the beginning of the analysis and then at regular intervals as a control of the drift or instability of the system. Also the tablet was mounted with a copper standard and carbon coated before being used.

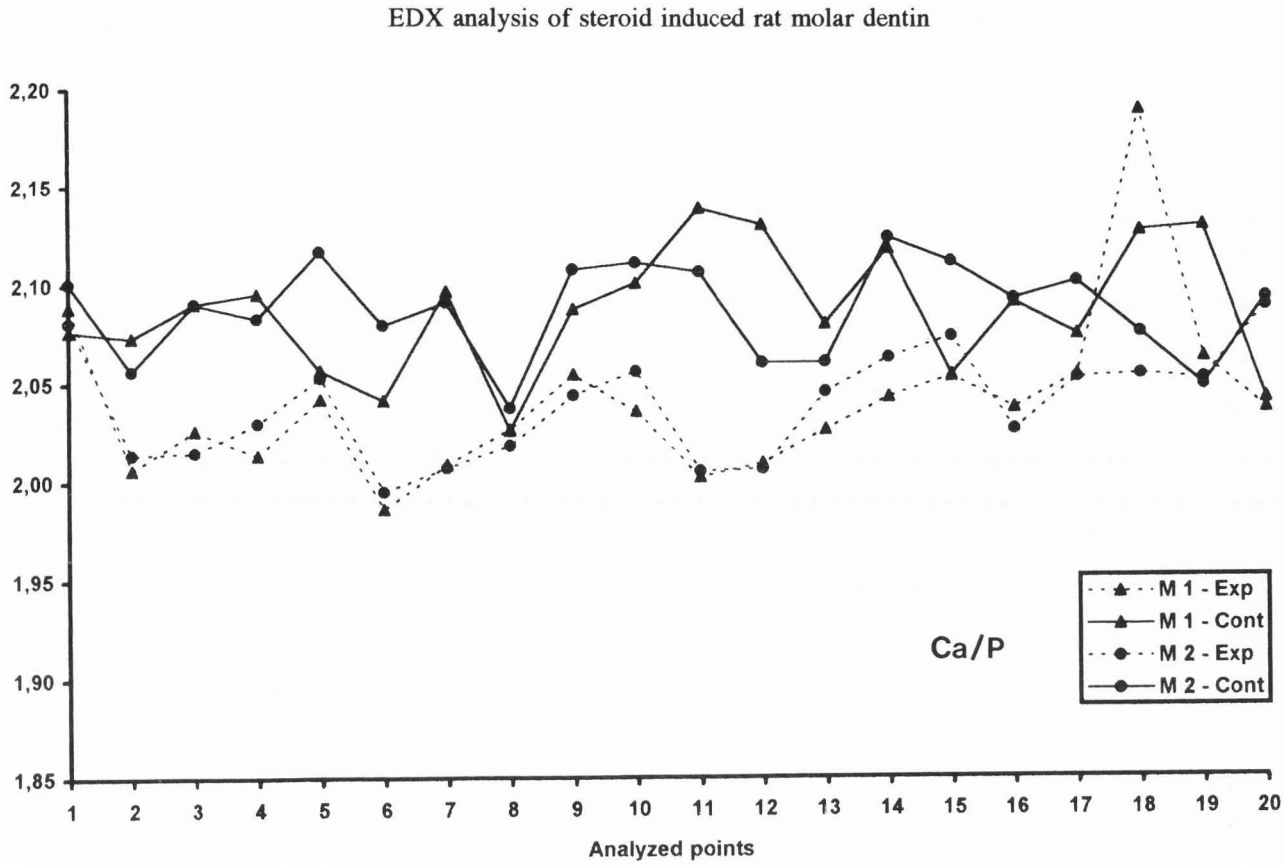


Figure 2. Mean values of calcium/phosphorus ratio in the analyzed points 1 to 20.

Spectra were collected at 20 kV. The estimated penetration depth for the electrons at 20 kV is 3 μm in bone and assumed to be about the same in teeth (Åkesson *et al.*, 1994; Engel, 1981). The incident electron beam current to sample was adjusted to obtain a preferred count rate of 5000 photons/second for Cu, which gave a count rate of approximately 2500 for teeth. Each spectrum was collected during 100 seconds live time.

Each section contained three molars; the anterior molar and the middle molar were used for analysis. The anterior molar had five roots and the middle molar four roots. In both molars, the most axially sawn root was chosen for locating the measuring points. In the dentin, three measuring points each were placed in a row along the pulp: in the buccal, mesial, lingual and distal aspect. Another measuring point in each direction was placed in the middle of the dentin, and one point below the dentin-cementum border, which made a group of five points in all in each direction (Fig. 1). To preserve equal geometric relations between analyzed points in different directions and the spectrometer, all sections were rotated in the SEM to get the anterior part of the specimen to the right and thus the buccal direction upwards. All points were individually placed and carefully adjusted to avoid cracks or other charging areas on the surface.

The detection limit for elements present, using the ZAF/PB program, is 0.1-0.5 weight % (wt%). After specimen preparation, the final concentrations of sodium (Na), magnesium (Mg), sulphur (S), aluminum (Al) and chlorine (Cl) were all below the concentration limit for reliable measurement in this program. All elements present were analyzed simultaneously and data are presented in wt%.

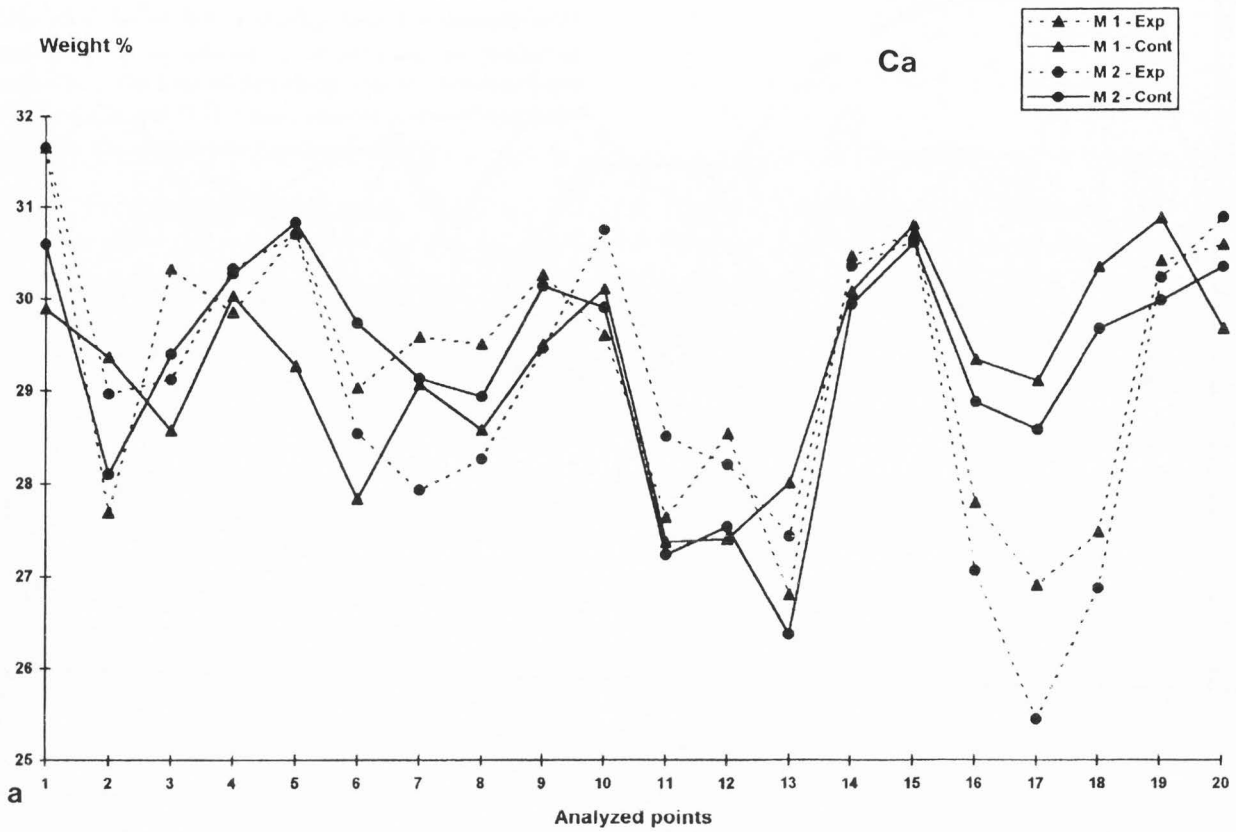
Statistical method

Analysis of variance with repeated measurements (Winer, 1971) was used for analysis of the Ca and P content. Differences were considered significant when $p \leq 0.05$.

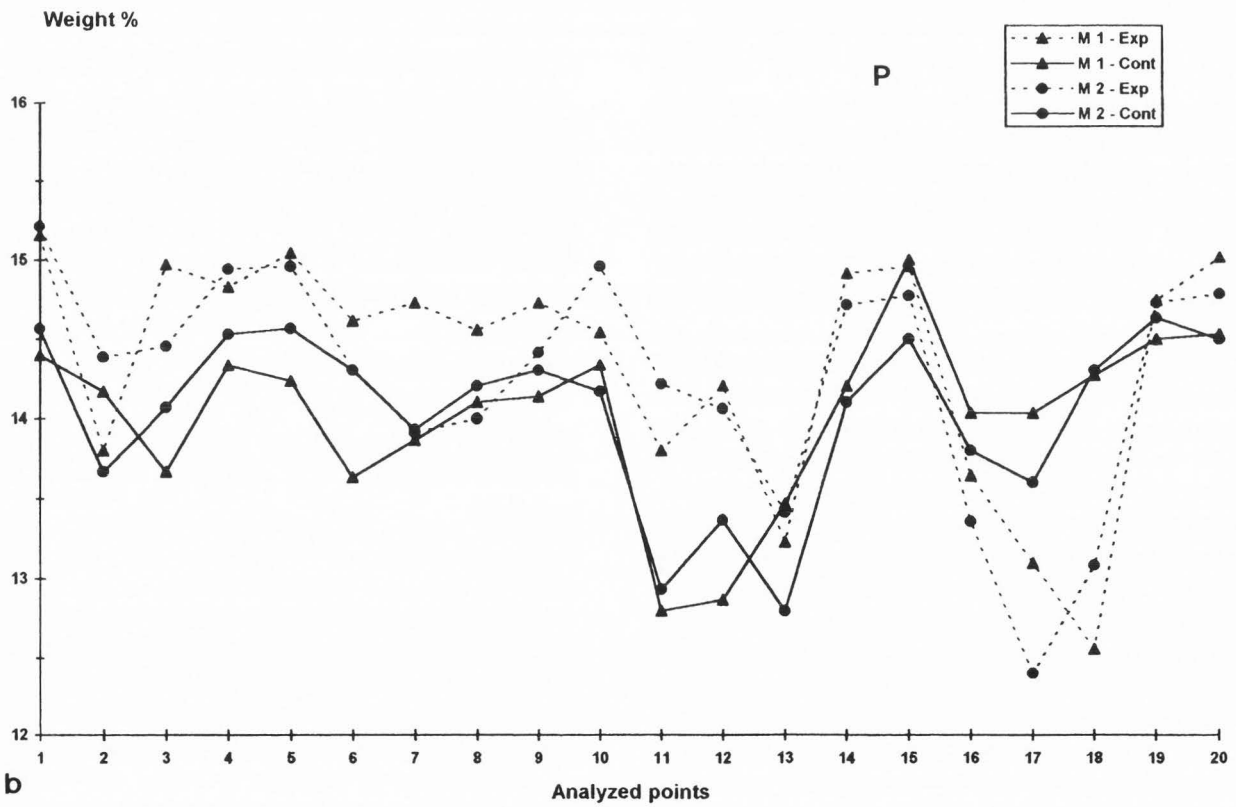
Results

No significant differences were found in the mean Ca/P ratio (wt%), between the group of points in different directions, or within the group of points in each direction. The mean Ca/P ratio was slightly above 2.0 in both the experimental and the control group (Fig. 2).

The EDX-analysis of the Ca and P content of the dentin showed no significant differences between the experimental and the control group concerning the overall


Weight %
Ca
M 1 - Exp
M 1 - Cont
M 2 - Exp
M 2 - Cont
32
31
30
29
28
27
26
25
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Analyzed points

a


Weight %
P
M 1 - Exp
M 1 - Cont
M 2 - Exp
M 2 - Cont
16
15
14
13
12
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Analyzed points

b

Figure 3 (on facing page). (a) Mean values of calcium (wt%) are shown in the analyzed points 1 to 20. In the experimental (n = 7) and control (n = 3) group, point 1 shows significantly higher values of calcium compared to points 2 and 3. At points 16-18, the values are significantly lower in the experimental group compared to the control group. (b) Mean values of phosphorus (wt%) are shown in the analyzed points 1 to 20. In the experimental (n = 7) and control (n = 3) group, point 1 shows significantly higher values of phosphorus compared to points 2 and 3. The lower values found in the experimental group at points 16-18 are not significantly different from the control group. M1: anterior molar; M2: middle molar; Exp: experimental; Cont: control.

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effect of treatment, although several interactions were found between points and treatment. Mean values of Ca and P at the analyzed points 1 to 20 are shown in Figures 3a and 3b. Within the group of three points close to the pulp in the buccal direction, significantly higher values of Ca and P were found when comparing the first point to the second (Ca: $p < 0.001$; P: $p < 0.05$) and to the third point (Ca: $p < 0.001$; P: $p < 0.01$) in both the experimental and control group. In the distal direction, significantly lower values of Ca ($p < 0.001$) were found in the experimental group compared to the controls at points 16, 17 and 18 (Tables 1 and 2).

Mean values of the three inner points close to the pulp in each direction were calculated for Ca and P. In the distal direction, significantly lower mean values of Ca were found in the experimental group ($p < 0.001$). In the buccal, mesial and lingual directions, no significant differences of the mean values of Ca were found between experimental and control groups. The mean value of P in the four directions showed no significant differences, but in the distal direction the value of P in the experimental group was lower than in the control group.

Discussion

In a previous experimental study on rat molars (Näsström *et al.*, 1989), it was shown that dentin developed during steroid treatment as a thin area along the root canal walls. To minimize the effect of incorrect positioning of the inner measuring points in this area and to ensure correct values of Ca and P, three points each were positioned in a row close to the pulp in four different directions. The mean value of Ca at these points in the distal direction showed lower values in the experimental group compared to the control group. The lower Ca value found in the distal direction might reflect corticosteroid induced mineralization, as part of the early

Table 1. Mean values of calcium in the three inner points of the anterior molar in the different directions.

Direction/ point no	Experimental group Mean	SD*	Control group Mean	SD*
Buccal				
1	31.7	2.1	29.9	1.2
2	27.7	1.8	29.4	0.6
3	30.3	1.0	28.6	0.7
Mesial				
6	29.0	1.2	27.8	2.0
7	29.6	1.3	29.1	2.1
8	29.5	1.3	28.6	0.7
Lingual				
11	27.6	2.0	27.4	0.8
12	28.5	1.6	27.4	4.0
13	26.8	1.1	28.0	1.7
Distal				
16	27.8	2.1	29.3	2.2
17	26.9	2.4	29.1	1.0
18	27.5	1.6	30.3	0.4

Table 2. Mean values of calcium in the three inner points of the middle molar in the different directions.

Direction/ point no	Experimental group Mean	SD*	Control group Mean	SD*
Buccal				
1	31.7	1.1	30.6	1.3
2	29.0	1.4	28.1	1.0
3	29.1	1.2	29.4	0.5
Mesial				
6	28.5	1.5	29.7	0.8
7	27.9	1.3	29.1	0.9
8	28.3	2.0	28.9	2.5
Lingual				
11	28.5	1.3	27.2	1.2
12	28.2	0.9	27.5	0.5
13	27.4	2.9	26.4	0.8
Distal				
16	27.1	1.5	28.9	1.2
17	25.4	1.1	28.6	0.8
18	26.9	3.0	29.7	1.1

*SD = standard deviation

incomplete mineralization front. This result may correspond to observations seen in patients who had received high amounts of corticosteroids, where a widened predentin zone with an irregular mineralization front is observed (Näsström *et al.*, 1993). The data could also be compared to enamel formation, where a higher rate of matrix formation has been shown at the mesial surface of developing enamel in maxillary rat molar cusps when compared with the distal surface (Caracatsanis *et al.*, 1989; Lange and Hammarström, 1984).

The ratio of Ca/P in rat dentin was equal, about 2.0, in both groups and at all points, which indicated deposition of Ca and P in a normal proportion between these two major constituents of hydroxyapatite (Engel and Hilding, 1984). The formula of hydroxyapatite is $[Ca_{10}(PO_4)_6(OH)_2]$, with a Ca/P ratio in human normal dentin estimated as 1.54 (Daley *et al.*, 1990). In developing mouse molars, the Ca/P ratio for dentin is between 1.8-2.3, increasing in value with the age of the mouse (Engel, 1981; Engel and Hilding, 1984).

Rat molars were chosen for this study because the normal development and maturation of dentin in the rat resemble that of other mammals (Bernard, 1972; Lange and Hammarström, 1984; Mjör, 1985). The method using EDX microanalysis allowed simultaneous quantification of multiple elements in thin undecalcified sections of teeth. The results could be evaluated for relative variation and related directly to anatomic areas of interest before and after microanalysis (Åkesson *et al.*, 1994; Green *et al.*, 1970; Hals *et al.*, 1988). The preparation of the specimen for EDX microanalysis, following the method of Donath (1987), included critical-point drying in order to induce minimal shrinkage and to preserve the interodontoblastic relations and the cell processes with the filling of the tubules (Jean *et al.*, 1986). The specimens were embedded in methylmethacrylate, ground to thin sections and carefully polished before being carbon coated to obtain the best conditions when SEM/EDX analysis was performed (Boyde *et al.*, 1986). The jaw sections contained three molars each and only the anterior and the middle molars were used for analysis, because most of the roots of these molars could be cut perpendicular to the long axis, which was impossible for the posterior molar due to the anatomy of the upper jaw.

In an earlier study (Näsström *et al.*, 1989), corticosteroids in high doses were given to rats and formation of new dentin could be seen in the roots of the molars. The amount of corticosteroids given postoperatively after organ transplantation in humans was extrapolated to the equivalent amount to rats after similar treatment for longest graft survival (Konrad and Husberg, 1979), and the maximum amount of corticosteroids thus found was intravenously administered in both the earlier and this study.

In the crown pulp under the cusps, normal secondary dentin develops under the stimulus of attrition (Hoffman and Schour, 1940) but abnormal formation of new dentin along the root canal walls in adult rat molars after steroid administration was found in an earlier study (Näsström *et al.*, 1989). The level of grinding of the specimen was chosen in accordance with those findings, i.e., below the furcation. Functional differences between crown and root odontoblasts in terms of quantity and quality of synthesized phosphoprotein have been described by Steinfort *et al.* (1989). It has also been observed that the cellular content and activity in the root odontoblasts in developing mouse molars were lower compared to the first differentiated odontoblasts in the crown pulp (Andujar *et al.*, 1991). These registered differences between crown and root odontoblasts might partly explain the reaction to altered hormonal levels concerning corticosteroids, where corticosteroids in high doses induced formation of new dentin along the root canal walls, which could not be explained by attrition or other functional disturbances in rat or human teeth (Näsström *et al.*, 1989, 1993).

In studies of orthodontic tooth movement in rats, it has been shown that the normal distal drift tendency of the rat molars must be considered when interpreting the results of force application to the teeth (Diaz, 1978). In the periodontal ligament (PDL), which is a connective tissue, remodelling processes in cementum and adjacent bone in mature teeth exposed to orthodontic tooth movement take place. These PDL cells contain high affinity receptors for several steroid hormones (Hellsing and Hammarström, 1991). Further, it has been found that cementoblasts react differently to mechanical pressure than osteoblasts and fibroblasts, which might explain the frequent occurrence of root resorption on the distal side of the rat molar roots (Kvam, 1972). These findings are important since our results showed changes in the dentin close to the pulp in the distal direction of the roots. There might be a connection between the resorption area on the distal surface of the root to the pulp via the dentin tubules, which could have influenced our results. In the material from a previous light microscopy study of vestibulo-lingually cut rat molar roots (Näsström *et al.*, 1989), a few small resorption zones could be seen on the distal side of the distal roots in both the experimental and the control group, when reexamining the sections for this study. The resorptions could be explained by the effect of the normal distal drift tendency in rat molar teeth (Diaz, 1978). Thus, the difference found between the experimental and the control group concerning the Ca content close to the pulp in the distal direction could not be related to the resorption zones on the distal root surface.

In conclusion, the results of this study showed that

the Ca/P ratio was similar in corticosteroid induced dentin compared to normal dentin. However, differences were found in the degree of mineralization in different directions of the root close to the pulp.

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This study was supported by the Swedish Dental Society and the Faculty of Odontology, University of Lund, Malmö, Sweden. Without the expertise of Dr. Peter Konrad, this work would not have been possible. We are especially grateful to Mrs. Birgitta Sluga and Mrs. Karin Mårtensson at the Dept. of Oral Pathology, Malmö, Sweden for all their laboratory help.

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Discussion with Reviewers

J. Wroblewski: What evidence do you have that chemical fixation, embedding and hydrous preparation of the specimens did not change calcium and phosphorus concentrations that are found *in vivo*?

S.H. Ashrafi: How much elements were lost during perfusion with glutaraldehyde and embedding in methylmethacrylate?

G.M. Roomans: The authors use aldehyde-fixed material, apparently assuming that Ca and P are so strongly bound that these elements are not released during fixation. Do the authors have evidence from own work or from literature for this?

Authors: In the study by Åkesson *et al.* (1994) (text reference), three different methods of analysis of elements in bone were compared. These three analysis techniques also involved different preparation methods. The preparation method of the specimens for EDX-analysis was the same as in the present study, namely methylmethacrylate (MMA) embedding, except that, in the present study, the specimens were first fixed with glutaraldehyde. The specimens prepared for neutron activation analysis (NAA) were defatted with a chloroform/methanol mixture. Inductive coupled plasma emission spectroscopy (ICPES) was carried out after dry ashing of the specimens. The results of Åkesson *et al.* (1994) showed very small differences between the three methods of analysis, but the Ca/P ratio was a little higher for the EDX-method, which might reflect a minor loss of P due to the preparation technique which involved an alcohol series with increasing concentrations before MMA embedding. It has been suggested by Nicholson *et al.* (1977), that Ca is more tightly bound to bone matrix than P, which might partly be washed out during preparation. In a study on mouse molars, Engel and Hilding (1984) (text reference) discussed the problem of aldehyde fixation and assumed that this treatment would not lead to loss or translocation of diffusible substances. On the contrary, they believed that the aldehyde fixation would cross-link the proteins in the matrix, so that the specimen theoretically would withstand carbon evaporation and beam damage better. The results of our study concerning the Ca/P ratio for dentin are in agreement with the results of Engel and Hilding (1984). Some P is probably washed out by alcohol and water and not by the aldehyde. This could provide a possible explanation why the P values did not differ significantly between the experimental and control group in our study.

J. Wroblewski: Could the differences between the elemental content of the corticosteroid induced dentin and normal dentin *in vivo* be lost during specimen preparation used in the present investigation?

Authors: In this study, we have performed no specific investigation as to whether the chemical binding of Ca and P is different in the mineralization process of corticosteroid induced dentin from the binding in normal dentin and thus could be more "sensitive" to preparation procedures. However, we find it unlikely that the difference between the experimental and control group in our study should be concentrated in the distal direction of the teeth and not in the whole area close to the pulp if the difference was due to preparation technique or to binding of Ca and P in the mineralization process.

G.M. Roomans: The authors use methacrylate as a matrix in the quantitative program of the microanalyzer system. Hydroxyapatite consists, in addition to Ca and P, of O, which also could have been used as a matrix. Do the authors feel that so much methacrylate penetrated into the dentin that it was more reasonable to take methacrylate as a matrix instead of O?

Authors: Biological tissues are not homogenous, and this creates a problem when they are analyzed. It is true

that for hydroxyapatite only, oxygen should be used as matrix. In this investigation, the predentin zone is of main interest. In this zone, the degree of mineralization varies. The matrix in unmineralized or partly mineralized cells is protein. In methacrylate embedded material, the matrix is composed of a locally unknown mixture of protein and methacrylate. The mean value for Z^2/A, which is used in the LINK ZAF/PB program for MMA, is 3.16, while the value of Z^2/A for oxygen is 4. The ZAF/PB program provides a Z^2/A value for protein matrix of 3.28. The value for MMA and protein matrix are very close, and therefore we chose MMA as matrix.

J. Wroblewski: Have you tried to analyze mineral content of dentin by an independent/other method?
Authors: We have not tried to analyze the mineral content of dentin by another independent method, because the results of the investigations concerning bone (Åkesson *et al.*, 1994) showed that the EDX method was as accurate and precise as the NAA and ICPES methods for Ca and P. We have been working with the same analysis equipment and used the same preparation technique as Åkesson *et al.* (1994).

S.H. Ashrafi: Why was only a copper standard used for calibration, instead of both aluminum and copper?
Authors: In the LINK system, only one gain calibration element is needed. The second calibration point in the low energy area is provided by the system itself, using a noise peak at zero keV.

G.M. Roomans: How thick is the layer of corticosteroid-induced dentin? Can it be resolved by the microanalysis and distinguished from normal dentin, taking into account the lateral resolution of the analysis?
Authors: In a previous study, Näsström *et al.* (1989) showed that corticosteroids induced dentin formation in rat molars. This was shown by tetracycline labeling and visualized by ultraviolet light microscopy. According to the micrographs in that paper, the width of the corticosteroid induced dentin formed could be estimated to be about 100 μm. The maximum diameter of the electron probe in the EDX analysis is estimated to be about 5 μm. When the measuring points are placed as close to the pulp as possible, we should be well inside of the region of corticosteroid-induced dentin.

J. Wroblewski: What is the possible cellular mechanism by which corticosteroids increase apposition of dentin?
Authors: The results of previous experimental studies on the effect of corticosteroids on dentin formation are contradictory. The dentin reaction to corticosteroids differ from excessive formation of bone-like substances in the pulp of rat incisors (Anneroth and Bloom, 1966), to no alteration of dentin formation after the same treatment in other studies (Ball, 1976, 1977). Studies on rat molars (Johannessen, 1964) and on rat incisors (Teixeira *et al.*, 1977) showed a depressed dentin deposition rate after corticosteroid administration. The amount of corticosteroids given, as well as the way of its administration, differs between the studies. In our study, the rats received high doses of intravenously administered corticosteroids corresponding to the doses given to renal transplant patients (Näsström *et al.*, 1985, 1989, text references). Our hypothesis is that it is necessary to use a high concentration of corticosteroids to be able to get enough of the drug to the receptors in the odontoblast cell and thus start the mineralization process resulting in the excessive formation of dentin seen in our studies.

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