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EFFECT OF FLUORIDE AND COBALT ON FORMING ENAMEL: SCANNING ELECTRON MICROSCOPE AND X-RAY MICROANALYSIS STUDY

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Abstract

The forming surfaces of enamel of rat incisors were examined by scanning electron microscope one hour after injection of either 5 mg/100 g body weight of sodium fluoride or 12 mg/100 g body weight of cobalt chloride. The cell debris from the surfaces of the separated incisors was either gently wiped off with soft facial tissues or chemically removed by treating with NaOH, NaOCl or trypsin. Best results to remove cell debris were obtained from 0.25% trypsin treatment.

SEM studies revealed that the surface of the normal secretory enamel was characteristic in appearance with well-developed smooth prism outlines. In fluoride specimens the prism outlines were feathery in appearance, laced with protruding spine-shaped clusters of mineral crystals. In the case of cobalt treatment, prism outlines were less uniform and in some areas they were incomplete.

The calcium concentration of surface enamel was significantly lower in the cobalt-treated specimens than those from control and fluoride-treated animals. The Ca:Mg ratio was also lower in cobalt-treated specimens as compared to control and fluoride-treated ones.

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Introduction

It is well known that exposure of forming enamel to certain ions both in vivo and in vitro inhibits the normal mineralization process in this tissue. Schour and Smith (1934), Eisenmann and Yaeger (1969), Walton and Eisenmann (1974), and Neiman and Eisenmann (1975) reported disturbances in rat incisor enamel formation following subcutaneous injections of fluoride and cobalt. Later, Bronckers et al. (1984a,b) showed that short term and long term exposure to fluoride created abnormalities in enamel formation in hamster tooth germs grown in organ culture. Bawden and Crenshaw (1984) reported a 50% reduction in calcium uptake by developing enamel in organ culture of rat molar tooth germs exposed to cobalt. Eisenmann et al. (1984) and Chen et al. (1986) reported changes in calcium distribution in secretory ameloblasts after fluoride and cobalt injection in rats. They also showed substantial accumulations of stippled material at the secretory regions of Tomes' process indicating interference with mineralization. In addition, SEM observation revealed differences in the surface morphology of distal ends of secretory ameloblasts in the cobalt-treated specimens as compared to controls. Ashrafi et al. (1987) found reduced concentrations of calcium near the distal surfaces of cobalt-treated, freeze-dried enamel organs. Thus, a model system has been described in which fluoride and cobalt each produce characteristic changes in secretory ameloblast morphology and function. Search for the mechanisms involved in this system led us to investigate further the effects of fluoride and cobalt on the surface morphology of the secretory enamel and the concentrations of calcium (Ca), phosphorus (P), and magnesium (Mg) in this region during a period of temporary interference with enamel mineralization.

Scanning electron microscopy (SEM) was used to study the forming enamel surface configuration and energy dispersive x-ray microanalysis to record concentrations of Ca, P and Mg.

Materials and Methods

Male Sprague-Dawley rats weighing 125-200 g were divided into three groups, each consisting of

three animals. The first group served as controls and received no injections (normal). The second group each received a single subcutaneous injection of 5 mg sodium fluoride per 100 g body weight, and the third group likewise each received 12 mg of cobalt chloride per 100 g body weight. After one hour the rats were killed by ether overdose and upper incisors with adherent alveolar bone were dissected free from the surrounding tissues. The incisor was manually dislocated from the socket causing a separation to occur between the enamel organ tissue and the forming enamel surface. The secretory region of enamel was demarcated under a dissecting microscope according to previously reported dimensions (Warshawsky and Smith, 1974). Enamel specimens were prepared separately for scanning electron microscopy and for x-ray microanalysis.

Scanning Electron Microscopy

Methods for the preparation of secretory enamel specimens for SEM involved the removal of cells and cell debris from the forming surface and tissue drying. Any cell remnants on the enamel surface were removed by gently wiping the surface with soft facial tissue or by treatment with either NaOH, 0.5% NaOCl or 0.25% trypsin solution. One set of specimens was kept under constant mechanical shaking for 2 to 24 h immersed either in 10% NaOH, 0.5% NaOCl or 0.25% trypsin. Another set of specimens was immersed in 0.25% trypsin solution for 2 h with occasional manual shaking. Following this surface treatment procedure, the secretory enamel was dissected free from the incisor (Fig. 1). A sketch was made of each specimen to help with orientation and in distinguishing the apical and incisal extremities while under SEM examination. The enamel specimens were dehydrated in a graded series of alcohols and critical point dried (Boyde and Martin, 1982). After drying, specimens were mounted on aluminum studs with permabond-910 adhesive. The specimens were coated with both carbon and gold. They were examined in a Cambridge Stereoscan S4-10 Scanning Electron Microscope at 45° tilt angle and 20 kV.

X-Ray Microanalysis

Specimens to be subjected to x-ray microanalysis were cleared of any cell remnants by gently wiping the enamel surface with soft facial tissue. The specimens were immediately frozen for 15 s in isopentane (-150°C) chilled with liquid nitrogen, transferred into liquid nitrogen, and freeze-dried at -35°C in a cryostat under vacuum for two days. After drying, the secretory enamel region was dissected from the incisor and mounted on carbon studs. The specimens were coated with a 20 nm thick film of carbon and examined in the scanning electron microscope, attached to an energy dispersive x-ray microanalyzer (EDAX Model 707A). The x-ray spectrometer was hooked to an 8K memory computer. The spectra were recorded in the range 0.0- 8 keV with 20 eV per channel. The operation condition of the analyzer was 100 s for recording spectra at 20 kV and 160 μA beam current. The tilt angle was 45° and take-off angle 30°. The beam size was around 100 nm. The calcium, phosphorus and magnesium peaks were recorded at 3.700, 2.02 and 1.26 keV, respectively.

The element counts per 100 s and peak-to-background ratios of calcium, phosphorus and magnesium were determined using a computer run program. A minimum of 12 readings for each animal group were recorded and samples from all animals were analyzed.

The freeze-dried forming enamel surfaces of the control, fluoride and cobalt treated animals were also analyzed by using a Philips Scanning Electron Microscope attached to an EDAX 9100-150 ECON Detector and an ECON-4-97-004 Windowless Detector.

Results

SEM examination of enamel surfaces cleaned by gently removing cell remnants with soft facial tissue revealed regions of varying quality. Some areas were free of debris, others showed cell remnants remaining attached to the forming enamel surface, and occasionally rows of prism walls were removed (Fig. 2). Exposure of forming enamel to NaOH or NaOCl for a period of 24 h under mechanical shaking conditions caused severe damage to prism walls and a smeared surface structure was observed. SEM examination of specimens treated with trypsin for 24 h showed complete removal of the ameloblast cell debris from the enamel surface and preservation of distinct prism outlines. Only the trypsin-treated specimens were used for SEM examination of the enamel surface morphology. By necessity, specimens treated only with gentle wiping were used for elemental analysis to avoid any loss of ions during chemical treatment. For these analyses, only areas with distinct prism outlines and with surfaces devoid of cellular debris were selected.

SEM examination of the forming enamel surface from fluoride-injected animals with 24 h treatment in trypsin demonstrated more distinct prism outlines than seen in normal, and cobalt-injected animals. The prism outlines in cobalt-injected animals were not uniform in arrangement and in some cases were deformed. Two hours treatment with trypsin under constant shaking produced better results. Distinct prism outlines were maintained in both normal (Fig. 3) and in fluoride-treated specimens (Fig. 4). However, in cobalt-treated specimens the prism walls looked as if they were collapsing (Fig. 5).

Trypsin treatment for 2 h with occasional shaking produced the best results. Sharp, uniform prism outlines were observed in normal (Fig. 6) and fluoride-injected specimens (Fig. 7). The surface of the prism wall in fluoride specimens showed a more feathery appearance as compared with controls. The cobalt-injected specimens showed deformed prism walls (Fig. 8).

Freeze-dried secretory enamel surface specimens of normal, fluoride and cobalt-injected animals were examined by x-ray microanalysis. Spectra were generated with a windowless ECON detector and with a standard beryllium window detector. Spectra from the windowless detector are shown in Figs. 9, 10, and 11 for normal, fluoride and cobalt-treated specimens, respectively. The spectrum obtained through the beryllium window detector for cobalt-treated specimens is shown in Fig. 12. In the case of the windowless detector, the x-ray spectra include
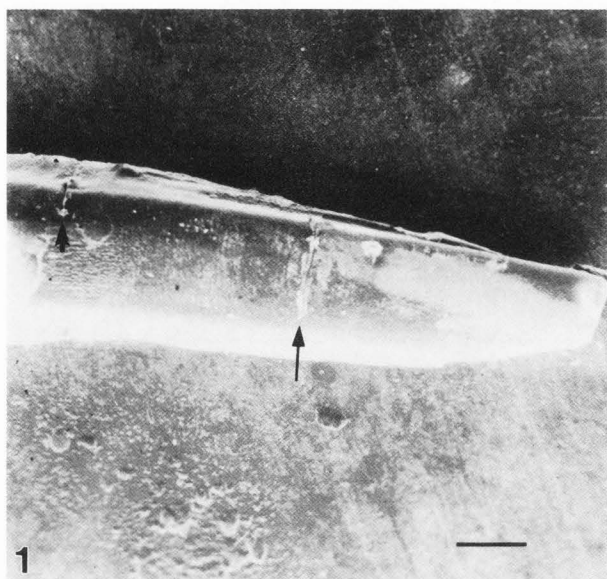
Fig. 1 SEM micrograph showing the rat upper incisor with marks 2 mm (arrow) and 4 mm (arrowhead) from the growing end. The region of secretory enamel for analysis was between the two marks. Bar = 500 μm
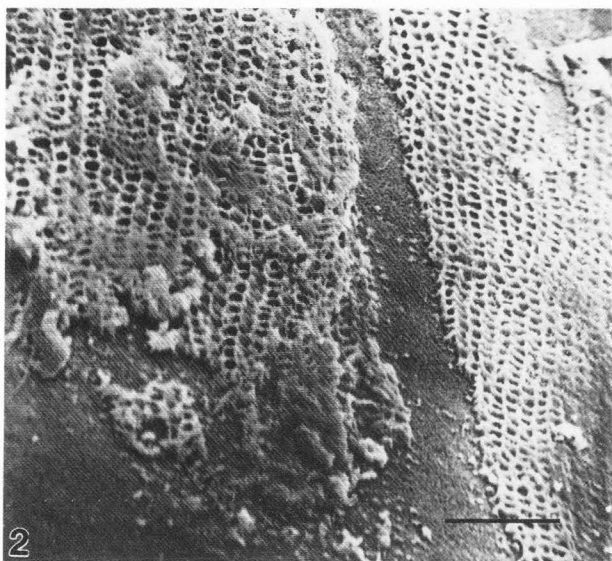

Fig. 2 SEM of forming enamel surface showing various appearances following cleaning, with cell debris remaining in some areas and prism patterns having been eliminated in others. Bar = 50 μm

Figs. 3,4,5 SEM micrographs of the forming enamel surface after 2 h trypsin treatment with mechanical shaking. Fig. 3 - Uniform, smooth prism outlines in normal specimens. Fig. 4 - Fluoride-treated specimens with forming prism outlines sharply outlined and feathery in appearance. Fig. 5 - Cobalt-treated specimens with collapsing prism walls. Bars = 10 μm
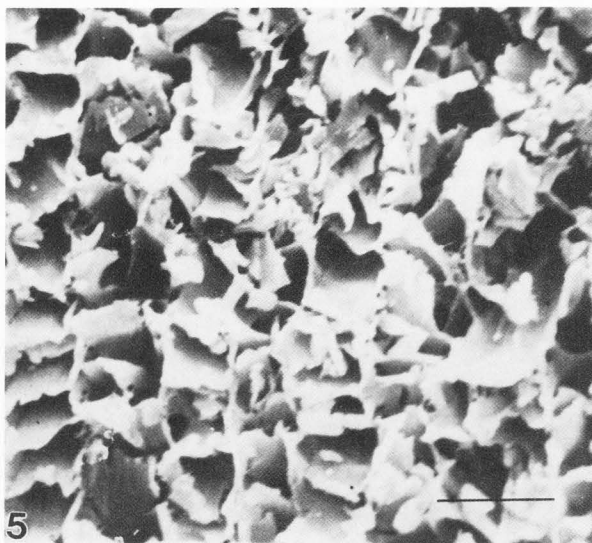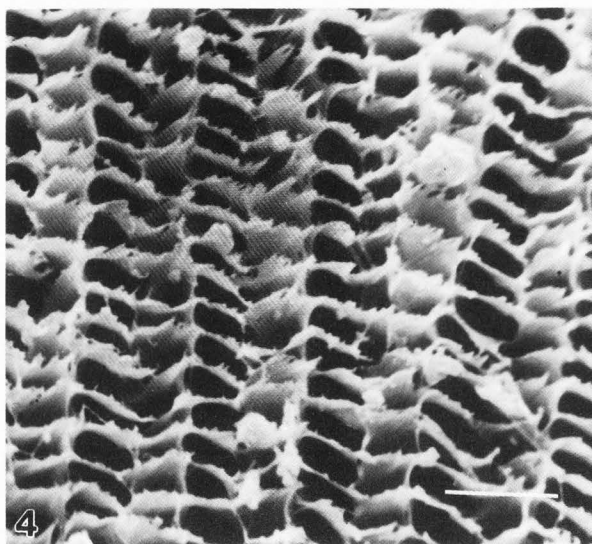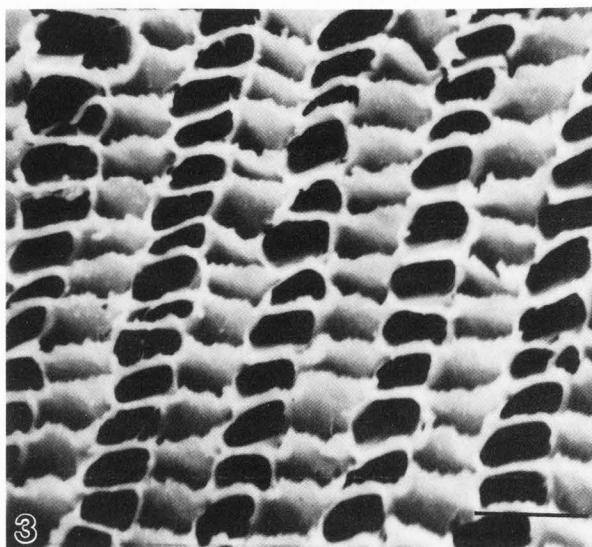
peaks for carbon, oxygen, sodium, magnesium, phosphorus, sulfur, chlorine, potassium and calcium. Similarly, representative spectra generated through the standard beryllium window detector showed peaks for these same elements except for carbon and oxygen. Both types of detectors produced clearly lower calcium peak heights in cobalt-treated specimens (Figs. 11 and 12) as compared to controls and fluoride-treated specimens.

Table 1 lists the element counts/100 s obtained through the beryllium window detector for calcium (Ca), phosphorus (P), and magnesium (Mg), for normal, fluoride and cobalt-injected specimens. These data clearly indicate lower concentrations of calcium and phosphorus in cobalt-treated specimens as compared to their normal and fluoride counterparts. Magnesium levels remain relatively stable.

Table 2 shows the means of peak to background (P/BG) ratios of elements recorded through the windowless detector for the forming enamel surfaces from normal, fluoride and cobalt-injected animals. Cobalt-treated specimens showed the lowest P/BG ratios for both P and Ca, with little change in magnesium.

Calcium/magnesium ratios for cobalt specimens were significantly lower than those from fluoride-treated and normal specimens. Thus magnesium levels became more prominent relative to calcium in the forming enamel surface under the influence of cobalt (Fig. 11).

Discussion

The well developed, distinct prism outlines observed on the surface of normal forming enamel were even more pronounced in fluoride-injected specimens. Prism outline as used in this paper refers to the wall of interprismatic region enamel which surrounds each pit into which prismatic enamel is being secreted. The wall area is secreted by the proximal secretory sites on Tomes' process, slightly in advance of secretion of the prism itself from the distal end of the process (Warshawsky, 1985). The feathery appearance of the prism walls in fluoride-treated specimens may represent mineral crystals. This excellent preservation of microstructure may have resulted from several factors. The studies of LeGeros and Tung (1983) revealed that incorporation of fluoride in vitro caused an increase in crystal size as compared to fluoride- free apatite. Fluoride is also known to promote hydrolysis of octacalcium phosphate (OCP) into apatite (LeGeros et al., 1984). The increased preservation of surface crystals may be further explained by the hypermineralization of surface enamel known to occur during the early stages of the fluoride
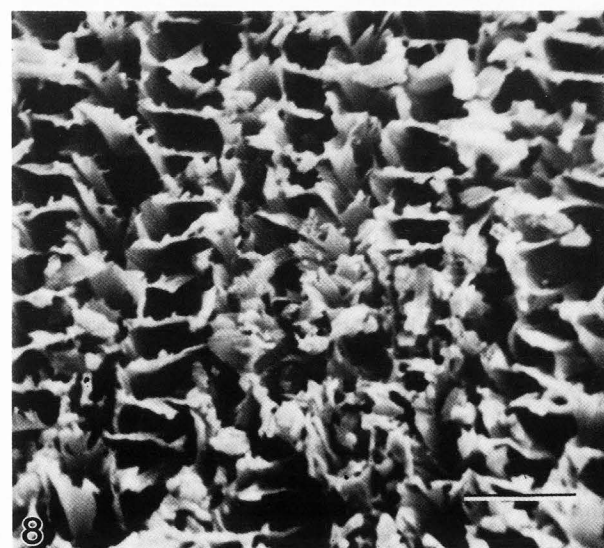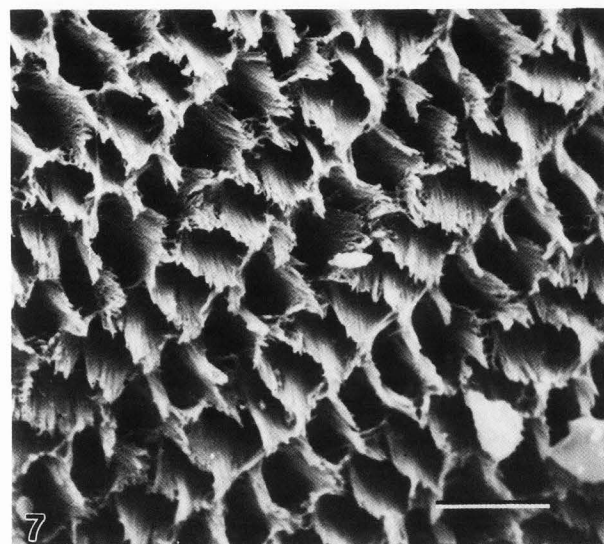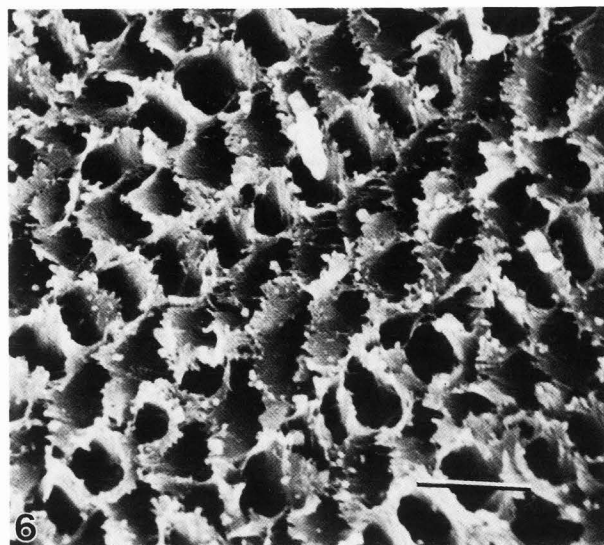
Figs. 6,7,8 SEM micrographs of the surface of developing enamel after 2 h trypsin treatment and occasional hand-shaking. Fig. 6 - Distinct formative surfaces and uniform outlines of prisms in controls. Fig. 7 - Fluoride-treated specimens with sharp, feathery prism outlines laced with protruding spine-shaped cluster of crystals. Fig. 8 - Cobalt-treated specimens with deformed prism walls. Bars = 10μm

Figs. 9,10,11 EDS spectra of freeze-dried forming enamel surfaces done through an ECON-windowless detector. Ca peaks in normal (Fig. 9) and in fluoride (Fig. 10) are higher than in cobalt-treated specimens (Fig. 11). Mg peak is more clearly visible in the cobalt than normal and fluoride-injected animals.

response (Neiman and Eisenmann, 1975). This phenomenon was confirmed by a significant increase in calcium/phosphorus ratio levels found in the x-ray analyses carried out in this study 1 h after injection of fluoride (Table 1).

The severely altered topography of prism outlines and pattern of distribution on the surface of forming enamel in cobalt injected animals may be a reflection of the severe disruption in enamel formation which occurs as a result of this insult (Neiman and Eisenmann, 1975; Eisenmann et al., 1984 and Ashrafi et al., 1987).

The use of windowless EDS analysis allowed a very graphic illustration of the fact that calcium and phosphorus levels decreased relative to the organic matrix during cobalt treatment, (Fig. 11). The reduced calcium and phosphorus concentrations in the cobalt-treated specimens correspond with previous studies demonstrating marked inhibition of enamel crystal formation in the early phases of the cobalt response (Neiman and Eisenmann, 1975). The reduced Ca/Mg ratio in these specimens raises interesting questions about the role of magnesium in enamel mineralization.

Magnesium is a minor constituent in enamel and has been associated with increased solubility in the progression of caries (Robinson, et al., 1981; Hallsworth, et al., 1972). In a recent review of magnesium in tooth enamel, Terpstra and Driessens (1986) pointed out that very little of this ion is within the apatite lattice and that most of it is either surface bound on the crystals, present in a separate mineral phase or
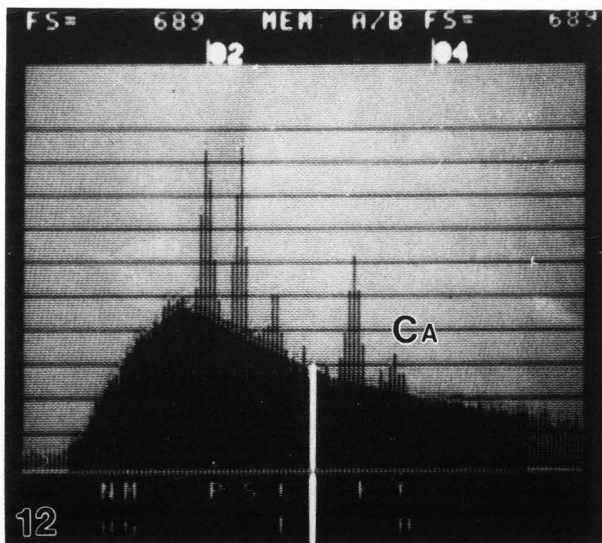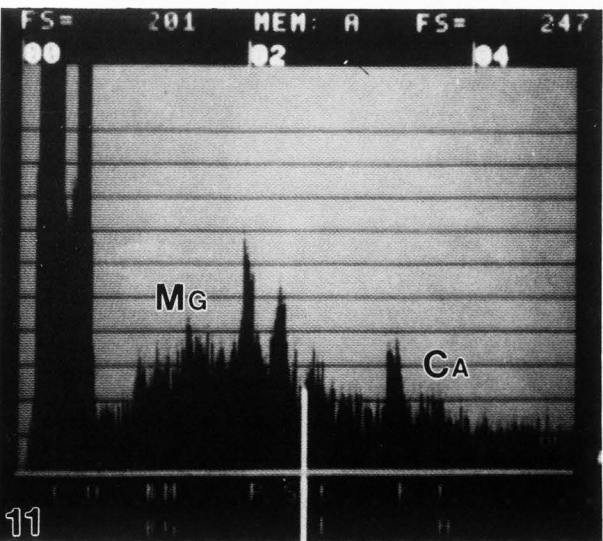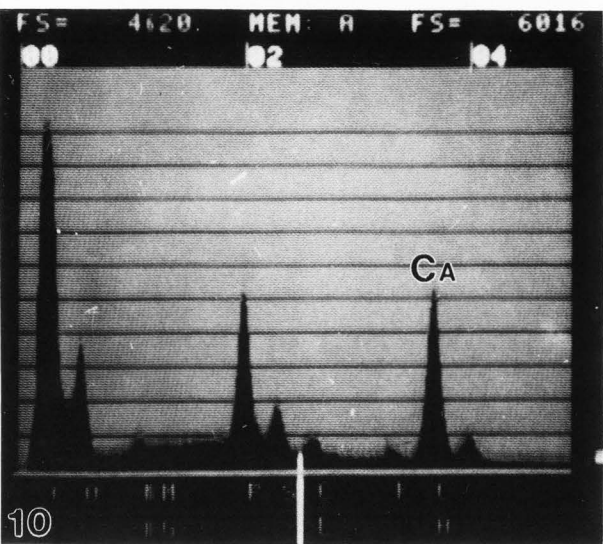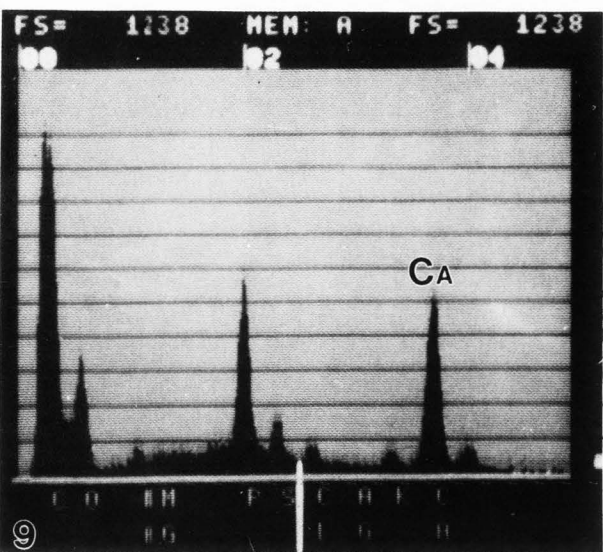

Fig. 12 EDS spectrum of the freeze-dried forming enamel surfaces performed with a standard beryllium window detector. Notice low Ca peak in cobalt treated-specimen.

TABLE 1. MEAN, SEM AND RATIO VALUES OF ELEMENT COUNTS PER 100 SECONDS FROM THE FORMING ENAMEL SURFACES OF NORMAL, FLUORIDE AND COBALT-TREATED SPECIMENS.

Specimen	Magnesium	Phosphorus	Calcium	Ca/Mg	Ca/P
Normal	99.6 ± 5.5	23,835 ± 437	35,748 ± 650	376	1.47
Fluoride	112.2 ± 8.3	21,203 ± 337	37,085 ± 979	349	1.77*
Cobalt	91.8 ± 5.0	*10,961 ± 966	*15,812 ± 1,462	185*	1.40

*Statistically significant from normal at P < 0.01

TABLE 2. EDS ANALYSIS OF THE FORMING ENAMEL SHOWING PEAK TO BACKGROUND RATIOS FOR Ca, P AND Mg IN NORMAL, FLUORIDE AND COBALT-INJECTED ANIMALS.

Specimen	Magnesium	Phosphorus	Calcium
Normal	0.05 ± 0.02	4.87 ± 0.11	13.66 ± 0.23
Fluoride	0.04 ± 0.01	4.97 ± 0.16	15.12 ± 0.46
Cobalt	0.06 ± 0.02	*1.23 ± 0.15	*0.69 ± 0.17

*Statistically significant form normal at P < 0.01

contained in the organic matrix. Magnesium is known to inhibit apatite crystal development (Bachra et al., 1965). It suppresses hydrolysis of octacalcium phosphate to apatite (LeGeros et al., 1984). In a study of the uptake of magnesium in developing bovine incisor enamel, Robinson et al., (1984) observed a marked increase in concentration during the transitional phase of enamel formation. They speculated that this might be related to the porous and highly-hydrated state of the enamel at this stage due to the replacement of the organic matrix by fluid. They also noted that changes in the cell morphology during transition from the secretory to the maturation stage could be a factor by altering the permeability of the enamel organ. It was stated that the role of magnesium in normal enamel development remains unknown.

It may be that the decreased calcium/magnesium ratio in our cobalt-treated specimens was related to altered secretory ameloblast morphology and function.and the concomitant accumulation of unmineralized stippled material. Changes in permeability of the ameloblast layer could allow free entry of various ions such as magnesium which might be taken up in the accumulating stippled material on the enamel surface while normal mineralization is inhibited. This poorly mineralized enamel surface is also very likely responsible for the altered surface morphology reported in this study.

The cobalt model system is of interest not only in searching for mechanisms of calcium transport via the ameloblasts during altered mineralization, but also for investigating the elusive role of magnesium in enamel mineralization. The prominence of this ion in altered surface enamel may be incidental to other changes or it may play a direct role in the inhibition of enamel crystal formation in this region of disturbed enamel mineralization.

Conclusions

1. Treatment with trypsin was the most effective method to remove cellular remnants from the enamel surface.

2. Fluoride treatment gave a sharp, feathery appearance to the surface extremity of the developing prism wall.

3. Cobalt-treatment produced a severely altered topography of the prism outlines and their pattern of distribution.

4. Cobalt-treated specimens displayed a lower surface calcium concentration than control and fluoride-treated specimens.

5. The Ca:Mg ratio in surface enamel from cobalt-treated specimens was lower than control and fluoride-treated specimens.

Acknowledgments

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Discussion with Reviewers

G.M. Roomans: I am puzzled by the figures provided in Tables 1 and 2. According to Table 1, the Ca/P ratio is not affected by cobalt treatment. In Table 2, the P/B value for Ca decreases with a factor of 20 versus that for P with a factor of 4. Unless the authors have made an error, this means that there was a factor of 5 difference between the backgrounds used for the P/B ratio for P and Ca. Where was the background calculated? In a peak-free region, under the peak, or over the entire spectrum?

Authors: Data illustrated in Tables 1 and 2 are different because two types of instruments were used. For Table 1 X-ray counts were generated and recorded through a Cambridge S4-10 Stereoscan Microscope hooked to a beryllium window EDAX detector unit. In Table 2 peak to background ratios were obtained through the Econo 4-97-004 windowless detector attached to a Philips Scanning Electron Microscope 515. The background was calculated in a peak-free region.

G.M. Roomans: You discuss the source of the Mg in the spectra. What is the source of the sulfur, chlorine and potassium peaks?

Authors: The source of sulfur, chlorine and potassium peaks are the glycoaminoglycans and carbohydrate/protein complexes (sulfate and carboxyl) of the forming enamel matrix, Engel, MB and Hilding, OH (1984) Mineralization of Developing Teeth. Scanning Electron Microscopy IV: 1833-1845.

G.M. Roomans: Could you demonstrate N in your windowless ED analysis?

Authors: Yes we can demonstrate nitrogen (N), however, carbon (C) was overlapping the peak of N in the spectrum.

R. LeGeros: Why would the cobalt system be needed to investigate the elusive role of magnesium in enamel mineralization? Why couldn't the role of Mg be investigated without the simultaneous presence of Co?

Authors: Cobalt is known to interfere with the mineralization process by inhibiting calcium transport. During this study cobalt was found to decrease the Ca/Mg ratio as compared to normal and fluoride treated animals. Therefore we are suggesting cobalt as a model to investigate the role of Mg in the mineralization process.

R. LeGeros: In Figs. 5 and 8, some material appears to be deposited in the Co-treated specimens, thus, obstructing the prism outlines. Are these deposits organic or inorganic in nature?

Authors: From Figs. 5 and 8, it is difficult to determine the organic or inorganic nature of the deposits on the specimen. However, in our transmission pictures, we have noticed stippled material (organic in nature) in the secretory zones of the Tomes' process, Ashrafi, et al. (1987).

R. LeGeros: Was the trypsin method of removing cell remnants used before, if so, by whom? If not, the authors should be commended for developing this method. Could this method be used in separating the organic phase from the inorganic in enamel, dentine or bone samples?

Authors: This is the first time that trypsin has been used to remove cell remnants from the enamel surface. We doubt that trypsin could be used to separate the organic phase from the inorganic in enamel, dentin, or bone samples, as it is a very mild enzymatic treatment.

Y. Kogaya: The authors have shown that the surface of the normal secretory enamel is characteristic in appearance with well-developed smooth prism outlines while in the case of cobalt treatment the prism outlines are less uniform and in some areas they are incomplete. However, in my opinion, one can not rule out a possibility that the deformed prism walls may result from artifacts during tissue preparation. It may be that in the specimens exposed to cobalt the forming surfaces of enamel become structurally fragile because of the reduction in calcium uptake by developing enamel. Therefore, the authors should add some TEM micrographs of the enamel forming sites including Tomes' process of the secretory ameloblasts corresponding to each experimental group.

Authors: Tissue preparation is not the cause of deformed prism walls. This phenomenon was also previously observed by transmission electron microscopy of altered enamel surfaces resulting from cobalt treatments, Neiman and Eisenmann (1975) and Ashrafi, et al. (1987).

Y. Kogaya: It is reported that calcium concentration increased gradually and continuously from the early to late enamel secretion regions, Sasaki T, Debari K, Garant PR (1987) Ameloblast Modulation and Changes in the Ca, P and S Content in Developing Enamel Matrix as Revealed by SEM-EDAX. J. Dent. Res. 66: 778-786. In your specimens, did you notice the changes in the calcium concentration of surface enamel?

Authors: We have analyzed only a representative area of the secretory enamel with no attempt to compare early and late regions of enamel secretion.