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Deposition of Calcium Phosphate into Dentin and Enamel as Shown by Radioautography of Sections of Incisor Teeth Following Injection of ^{45}Ca into Rats

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To find out whether the calcium phosphate of dentin and enamel is elaborated in the cells prior to passing into the organic matrix or is initially deposited into the matrix, rats were injected intravenously with ^{45}Ca and killed 30 sec or 5 min later by glutaraldehyde perfusion; semithin sections of the undecalcified incisor teeth were then radioautographed for detection of the incorporated ^{45}Ca. Rat incisor teeth were selected since there is evidence that the calcium they take up is "stable"; that is, not subject to significant loss by exchange or by other physiocochemical processes. When *dentin* is examined after ^{45}Ca injection, the maximum radioautographic reaction is observed next to the junction with predentin and a gradual decrease up to the dentin enamel border. No radioactivity is detected in odontoblasts. These observations are interpreted as indicating that the initial site of calcium phosphate deposition is in the matrix of dentin. In the *enamel*, the radioautographic reaction is spread fairly uniformly throughout the matrix, with a weak reaction over ameloblasts attributed to radiation scatter. The interpretation is again that calcium phosphate is deposited into the matrix; and, furthermore, that this deposition begins as soon as the matrix is laid down and continues at about the same rate up to an advanced stage of mineralization.

Key words: Radioautography — Mineralization — Incisors — Odontoblasts — Ameloblasts.

Introduction

Radioautography has been used after injection of ^{32}P-phosphate or ^{45}Ca to identify the sites of mineral deposition in teeth. The publications on the subject[1] have usually led to the conclusion that label appears within the matrix of dentin and enamel rather than in the associated cells.

However, in these investigations, the teeth were not decalcified and the section thickness varied from 5 to 100 μm and more, so that the quality of the radioautographs was not as good as it might be. Since it is now possible within certain limits to cut semithin (1 μm) sections of undecalcified teeth embedded in plastic, it was decided to use such sections to re-examine the sites of deposition of minerals. The decrease in section thickness should result in much improved

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1 The deposition of label in tooth matrices was reported after injection of either ^{32}P-phosphate (Bélanger and Leblond, 1950; Leblond *et al.*, 1950; Leblond *et al.*, 1955) or ^{45}calcium (Bélanger *et al.*, 1954; Engfeldt *et al.*, 1954; Myers, 1955; Kumamoto and Leblond, 1956; Bélanger, 1957; Engfeldt and Hammarlund-Esler, 1957; Reith and Cotty, 1962; Suga and Murayama, 1965; Hammarstrom, 1967; Starkey, 1971).

resolution (Gross *et al.*, 1951). ^{45}Calcium was the isotope of choice since it emits electrons with a shorter pathway and, therefore, allows a better localization than ^{32}P-phosphate. Calcium was also preferred from a biological point of view, since it is less implicated than phosphorus in cellular activities unrelated to calcification.

To make sure that the initial sites of reaction would be detected, the rats used were killed early, i.e., 30 sec or 5 min after an intravenous injection of ^{45}Ca. The tooth selected for study was the incisor, for there is evidence that this tooth retains the incorporated calcium without significant loss by exchange or desorption (Carlsson, 1952; Bauer and Shtacher, 1968).

Materials and Methods

Location of ^{45}Ca in Incisor Tooth Dentin

Young male Sherman rats weighing 50–55 g were anesthetized with ether and given a single intravenous injection of 10 μCi of $^{45}CaCl_2$/g body weight (s.a., 10–20 mCi/mg) in a volume of 0.20 or 0.25 ml. Two animals were killed after 30 sec, three after 5 min, and two after 24 h following the injection, using intracardiac perfusion with glutaraldehyde fixative. A Venocath 16 plastic catheter was introduced into the heart through a puncture of the left ventricle and pushed to the beginning of the aorta. The catheter was connected through a Venopack with a bottle containing 2.5% glutaraldehyde in phosphate buffer (pH, 7.2) with 1g sucrose added per 100 ml to raise osmolarity and 0.5 mM $CaCl_2$ to assist in washing-out free labeled calcium ions. The perfusion set was mounted in such a way that the flask containing the fixative was about two meters above the operating board, so that the fluid flowed into the animal by gravity. After 15–20 min, perfusion was stopped and the incisor teeth removed from mandible and maxilla, trimmed and, without passing through a buffer wash, postfixed for one hour in cold 1% osmium tetroxide in phosphate buffer. The specimens were immediately dehydrated through a series of graded acetone solutions and, after infiltration in Epon-acetone mixtures (1:1 and 3:1), embedded in pure Epon and placed in an oven at 60° for 3 days to polymerize.

The sectioning of the undecalcified specimens was carried out near the proximal end (that is, the growing end) of the incisor tooth, using glass knives on a Porter-Blum microtome. Three or four 1-μm sections were aligned on a histological slide. Radioautography was done by the coating technique (Kopriwa and Leblond, 1962). Slides were dipped into melted NTB2 emulsion and stored at 40°. After rather short exposure (2 h–1 day) for the 24-h groups and a long exposure (18 h to 10 days) for the 30 sec and 5-min groups, the preparations were developed and fixed. They were then stained for 1 h in a 1% toluidine blue solution in 1% sodium borate. After drying, the slides were mounted in Permount resin and examined in the light microscope.

The observation of radioautographs was strengthened by counts of silver grains under oil immersion, with the help of a Whipple-type micrometer disc (showing a square divided by horizontal lines into 10 equal rows). The *baseline* of the square was aligned over the predentin-dentin junction, and the number of grains counted in successive rows until the whole thickness of dentin was covered. At the next step, the stage was moved so as to align the *top* of the square with the predentin-dentin junction, so that the rows now overlay successive regions of predentin and the nearby odontoblasts. Counting over dentin, predentin and odontoblasts was repeated over 60 areas above and below the predentin-dentin junction. The results were expressed per 106 μ^2 area (Table 1).

Location of ^{45}Ca in Incisor Tooth Enamel

The same preparations were used to examine enamel and ameloblasts. Silver grains were counted as for dentin except that the baseline of the square in the micrometer disc was aligned over the enamel-dentin border for grain counts over the enamel and the top of the square over the border between enamel and ameloblasts for grain counts over ameloblasts. Again, counting was repeated over 60 areas and the results expressed per 106 μ^2 area (Table 2).

Table 1. Radioactivity of dentin and associated structures in incisor teeth of rats injected with ^{45}calcium intravenously

Time of death after injection		30 sec	5 min	1 day
Exposure		36 h	18 h	2 h
Background count (per 106 μm^2)		0.7	0.4	0.2
Distance from predentin-dentin junction (μm)		Grain counts/unit area (minus background)		
175	dentin	—	—	4.5
165	,,	—	—	2.2
155	,,	—	—	2.0
145	,,	—	—	1.7
135	,,	—	—	1.0
125	,,	—	—	1.1
115	,,	—	—	1.4
105	,,	—	—	1.2
95	,,	—	—	1.0
85	,,	—	—	1.8
75	,,	—	—	1.5
65	,,	—	2.5	2.4
55	,,	—	2.3	2.2
45	,,	—	3.3	4.0
35	,,	4.0	3.4	7.4
25	,,	4.1	3.6	15.7
15	,,	4.9	3.7	14.6
5	,,	11.0	9.0	6.1
——	predentin-dentin junction	——	——	——
— 5	predentin	2.9	2.6	1.8
—15	,,	0.3	0.4	0.9
—25	odontoblasts	1.2	0.9	0.5
—35	,,	0.0	0.3	0.1
—45	,,	0.0	0.6	0.2
—55	,,	0.3	0.3	0.0
—65	,,	0.0	0.5	0.2
—75	,,	—	0.2	—

Isolated Odontoblasts

In another experiment, a 45-g rat received an intravenous injection of 10 μCi ^{45}Ca/g body weight and was killed by perfusion 5 min later. The right lower incisor was processed, sectioned without decalcification and radioautographed, using the same procedural steps as above. The left lower incisor, however, was broken to allow the removal of the pulp and odontoblasts with forceps; these tissues were then processed for radioautography at the same time and under the same conditions as the sections of the other incisor. Counts were made of the numbers of silver grains over the odontoblasts present in the tooth sections as well as over the cells isolated before processing (Table 3).

Effect of "cold" Calcium Treatment on the Uptake of Labeled Calcium by Dentin

It was decided to examine whether the amount of radiocalcium taken up by the incisor tooth would be decreased by administration of a large dose of nonradioactive calcium ions. Four littermate Sherman rats were weaned and placed on a regular pellet diet. Two days later,

Table 2. Radioactivity of enamel and associated structures in incisor teeth of rats injected with ^{45}calcium intravenously

Time of death after injection		30 sec	5 min	1 day
Exposure		36 h	18 h	2 h
Background count (per 106 μm^2)		0.7	0.4	0.2
Distance from border between enamel and ameloblasts (μm)		Grain counts/unit area (minus background)		
85	ameloblasts	0.1	—	—
75	„	0.9	0.2	—
65	„	0.3	0.2	—
55	„	0.6	0.2	—
45	„	0.3	0.6	—
35	„	0.6	0.5	—
25	„	0.9	0.4	0.8
15	„	0.5	0.3	0.7
5	„	1.5	0.8	2.5
− 5	enamel	2.8	1.5	8.0
−15	„	5.3	3.8	9.4
−25	„	6.3	4.6	9.1
−35	„	6.3	3.6	6.8
−45	„	5.8	2.8	8.4
−55	„	5.3	2.4	9.0
−65	„	—	1.8	8.2
−75	„	—	2.0	7.7
−85	„	—	—	5.8

Table 3. Grain counts over odontoblasts in radioautographs obtained 5 min after intravenous ^{45}Ca injection (10 μCi/g body weight; 1-day exposure)

	No of silver grains per unit area (× 100)		
	Odontoblasts	Background	P value
Odontoblasts within tooth section	91.7 ± 5.2	45.8 ± 5.2	$P<0.01$
Isolated odontoblasts	28.4 ± 2.2	25.8 ± 3.1	$P=0.3$

the animals which by then weighed 42 g, received a single dose of 10 μCi of ^{45}Ca/g body weight intravenously. One and a half hours later, two of the animals used as controls were given by stomach tube 1 ml of saline and the two others, used as experiments, 1 ml of a 2.5% calcium gluconate solution in distilled water. This treatment was repeated 18 h later. The animals were killed by intracardiac perfusion with 2.5% glutaraldehyde 24 h after the radioactive calcium injection. Radioautographs were prepared and silver grains counted over 60 areas of dentin (Table 4).

Results

Dentin

Radioautographs of cross sections taken near the growing end of lower and upper incisor teeth from animals sacrificed *30 sec* after intravenous injection of

Table 4. Influence of "cold" calcium administration on the amount of radioactive calcium retained in dentin 24 h after intravenous injection of ^{45}Ca (10 μCi/g body weight; 2 h exposure)

Distance from predentin-dentin junction (μm)	5	15	25	35	45
	Grain counts per unit area of dentin				
Saline treated controls	5.0	10.1	7.0	4.2	5.3
	4.6	13.4	10.1	5.4	3.5
	4.8	11.7	8.5	4.8	3.4
2.5% calcium gluconate treated	3.9	9.7	7.0	3.9	2.7
	6.4	12.5	7.2	4.0	3.2
	5.1	11.1	7.1	4.0	2.9

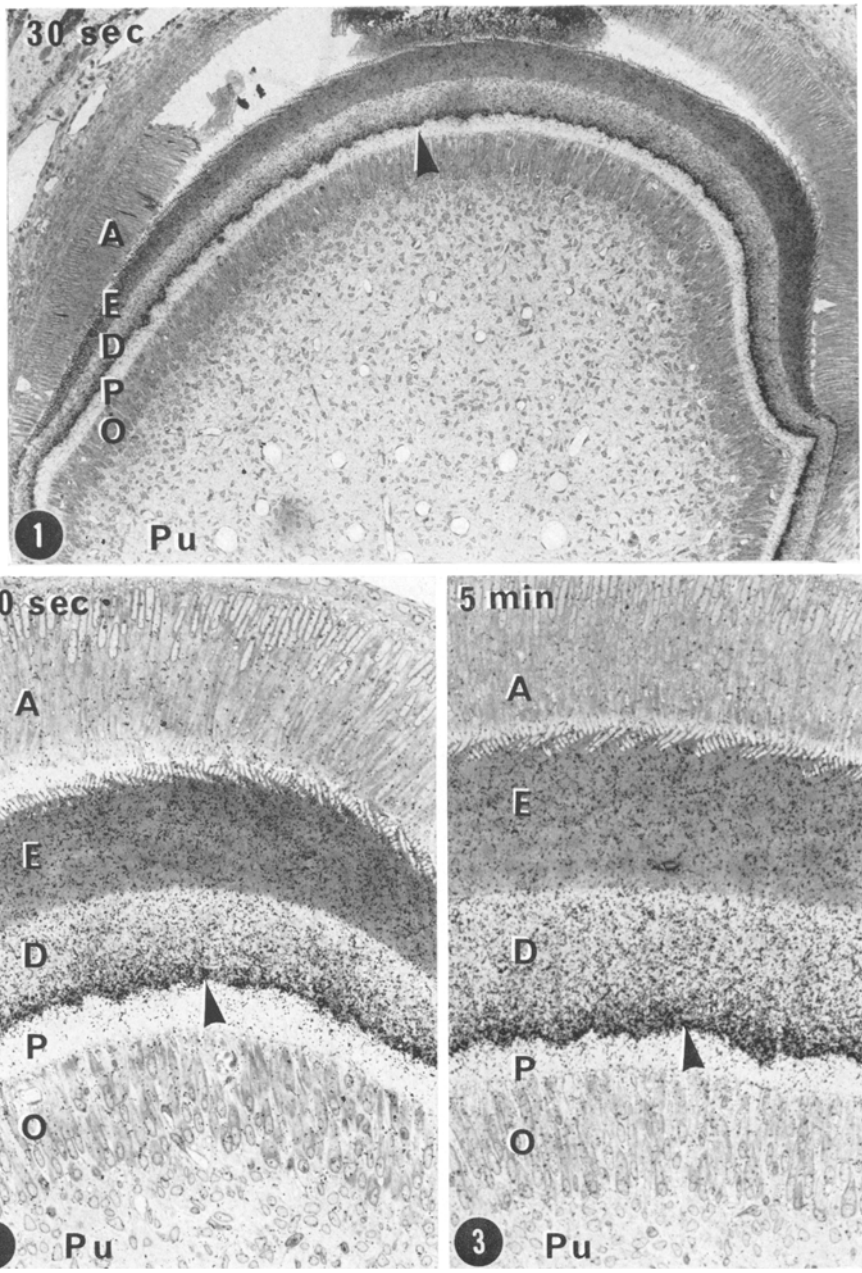
Analysis of variance showed a significant difference between counts at various distances from predentin-dentin junction ($P<0.01$), but no difference between saline and 2.5% calcium gluconate treated groups. Interaction between the two variables was not significant.

^{45}Ca showed a band of reaction along the edge of dentin next to the predentin-dentin junction (arrowhead, Fig. 1). Under high power, the reaction band of dentin appeared intense next to the predentin-dentin junction and decreased gradually toward the dentin-enamel border (Fig. 2). Few silver grains were present over predentin and even fewer over odontoblasts and pulp.

In the animals killed *5 min* after ^{45} Ca injection, the dentin pattern was the same. A reaction band outlined the edge of dentin, while a gradient of reaction extended over the rest of this structure (Fig. 3).

For the examination of the results at the *24-h* time interval, the section selected for illustration (Fig. 4) was taken farther from the growing end of the tooth than in that used for the 30-second and 5-min intervals. A reaction band was seen in dentin about 18 μ from the predentin-dentin junction. Under high power (Fig. 5), reaction gradients could be seen on both sides of the band, a long gradient extending in the direction of the dentin-enamel junction and a short gradient towards the predentin-dentin junction (corresponding to the amount of new dentin deposited over the 24-h period). Few silver grains were seen over predentin, odontoblasts and pulp.

The quantitative assessment of the reaction was carried out by counting silver grains in the teeth of three animals which had been injected with radiocalcium at the same; time the tooth sections were exposed for the durations indicated in Table 1. On the assumption that the numbers of grains were roughly proportional to exposure (Kopriwa and Leblond, 1962), the counts were corrected for a 36-h exposure. Thus, since the teeth from 24-h animals had been exposed for 2 h, the counts were multiplied by $^{36}/_{2}$. The corrected counts were plotted in Fig. 6 on a logarithmic scale. At 30 sec and 5 min after injection, the results showed a peak on the dentin side of the predentin-dentin junction corresponding to the bands in Figs. 2 and 3 respectively. Beyond the peak, the grain count decreased gradually up to the dentin-enamel border. On the predentin side of the peak, the counts showed a sharp reduction, which approximately followed a straight line. The reaction over



Figs. 1—3. Toluidine blue stained, coated radioautographs of incisor teeth of young rats killed soon after an intravenous injection of ^{45}Ca. The photographs depict undecalcified cross sections in the proximal region (growing end) of incisor teeth. *A* ameloblasts; *E* enamel; *D* dentin; *P* predentin; *O* odontoblasts; *Pu* pulp. (10-day exposure). Fig. 1. Thirty seconds after injection. The arrowhead points to a radioactive band over dentin next to the predentin-dentin junction. Predentin seems to be unreactive. A uniform reaction is seen over enamel, × 100. Fig. 2. Higher magnification of a region of the tooth shown in Fig. 1. From the radioactive band of dentin (arrowhead) a decreasing gradient of reaction extends to the dentin-enamel border. Few grains are seen over predentin and odontoblasts. On the enamel side, the reaction is diffuse, × 250. Fig. 3. Five minutes after injection. The pattern is similar to that observed at the earlier time interval (Fig. 2), × 250

odontoblasts was low but usually above background (Table 1). At 24 h, the radioactivity was intensified and the peak was within the dentin at some distance from predentin. A sharp fall on the predentin side of the peak roughly followed a straight line, whereas a gradual decrease on the other side was followed by a short rise just before the dentin-enamel border.

Enamel

At all time intervals, the reaction extended over the whole enamel without any distinguishable band (Figs. 1—5).

The *quantitative results*, which were obtained in the same animals as for dentin (Table 2), showed a generally uniform reaction throughout the enamel (Fig. 7). The moderate decrease toward the dentin-enamel border in the 5-min animal was not observed when the experiment was repeated and was not considered significant. On the other hand, a drop in the counts was regularly observed over the apical region of ameloblasts (Table 2) and approximated a straight line at the three time intervals (Fig. 7).

Significance of the Apparent Cellular Reactions

To find out whether the low grain count over odontoblasts was due to their uptake of labeled calcium or to scattered radioactivity emanating from dentin, these cells were separated from the lower left incisor prior to radioautography, whereas they were left within the tooth on the other side. The results (Table 3) indicated that the grain count was about twice as high as the background over the odontoblasts kept within tooth sections, but did not differ significantly from background in the case of the isolated odontoblasts. Hence, under the conditions used, no detectable amount of labeled calcium was retained by the cells.

Test of the Stability of the Label Taken up by Dentin

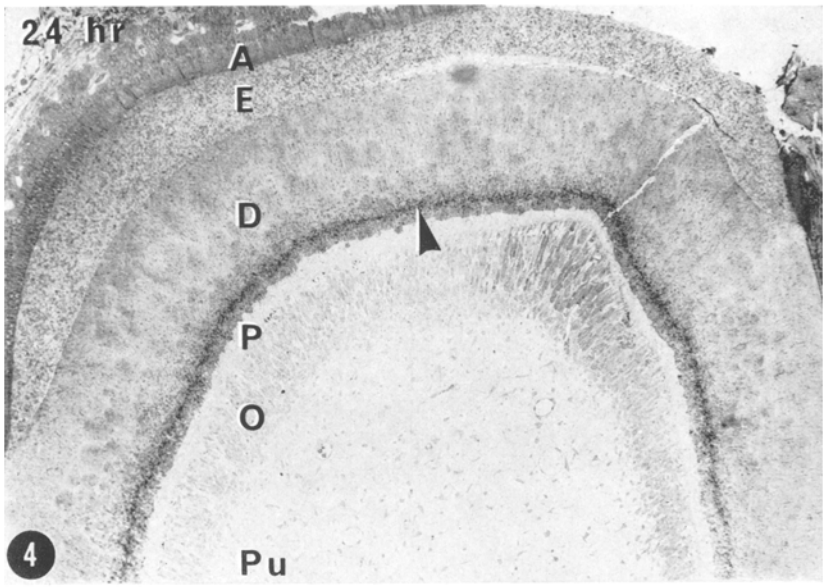
At 1.5 and 18 h after an injection of radiocalcium, two rats received large doses of "cold" calcium gluconate. When the amounts of radioactivity present in dentin were compared by radioautographic grain counts to the amounts present in two control animals given saline at the same times, the results (Table 4) raised the possibility of a slight decrease in the calcium gluconate treated group, but the analysis of variance showed that the difference was not significant. It was concluded that little, if any, of the radioactive calcium taken up by dentin was removed by treatment with large doses of "cold" calcium.

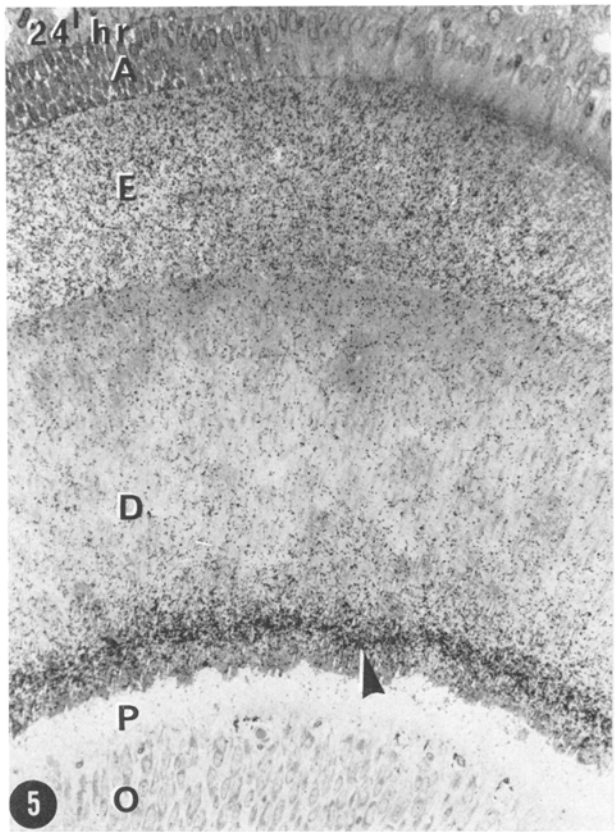
Discussion

Three main facts were observed in radioautographs of the growing ends of rat incisors after an intravenous injection of ^{45}Ca: immediate appearance of radioactivity within dentin and enamel; predominance of dentin radioactivity along the predentin border; and interstitial deposition of radioactivity in enamel. Our interpretation of these three facts follows.

1. Immediate Appearance of Radioactivity in Radioautographs of Dentin and Enamel

To avoid the possibility that ^{45}Ca be taken up by the tissues after death, the animals injected with the isotope were killed by intracardiac perfusion of a





Figs. 4 and 5

glutaraldehyde solution to which free calcium ions had been added. In this manner, biological phenomena were arrested instantly and the uncombined ^{45}Ca ions were washed out. Hence, the pattern observed in the animals killed 30 sec after ^{45}Ca injection (Fig. 2) truly depicted the sites of label uptake in the course of these 30 sec.

Nature of the Radioactive Material Present in Radioautographs

The ^{45}calcium responsible for the radioautographic reactions of dentin and enamel resisted extraction by the neutral fixative and processing fluids used in preparing the specimens, but was readily removed by treatment of the sections with dilute acids or ethylenediamine tetraacetate (unpublished). These properties coincided with those of the calcium phosphate of hard tissues. It was concluded that the radioautographic reactions were due to ^{45}Ca combined in the form of calcium phosphate.

Exchangeable and Stable ^{45}Calcium in Incisor Teeth

Chievitz and Hevesy (1937) first noted that the labeled ^{32}P-phosphate ions taken up by hard tissues could be either "stable" (through incorporation into newly-formed calcium phosphate) or "labile" (through uptake by adsorption or exchange with surface ions and subsequent loss by desorption or reverse exchange with non-radioactive ions). Over the years, the opinion arose that, when animals were sacrificed soon after injection of labeled mineral precursors such as ^{32}P-phosphate or ^{45}Ca, the uptake by hard tissues was due to adsorption or exchange rather than to new formation of stable calcium phosphate, whereas "in longterm experiments, growth and bone formation dominate skeletal deposition..." (Neuman and Neuman, 1958). Thus, Myers (1955) stated that, when his animals were killed within hours of injection of a labeled precursor, the growth of the tooth over that period was negligible; and, therefore, the uptake of label should be attributed to adsorption or exchange. When Triffitt *et al.* (1968) killed 80–100 g rats 1.5 h after an injection of ^{45}calcium and found that the amount of label entering the tibial diaphysis constituted 1.4% of the calcium present, they presumed that growth played no part in the uptake. Yet, in our experience, rats of this size gain about 5% of their weight per day. Such a gain, if uniformly distributed throughout the day, would increase body weight by 0.31% in 1.5 h. Assuming that the calcium content of bone increases at the same rate, the fraction of the entering calcium attributable to growth would be $(0.31 \times 100)/(1.4) = 22\%$; that is, a far from negligible amount. Briefly then, at least some of the calcium entering bones and teeth over short periods of time is added for growth.

In 1955, Carlsson found that the fraction of the calcium taken up by a process of exchange was large in the femur, but small or absent in the incisor tooth of the

Figs. 4 and 5. Toluidine blue stained, coated radioautographs of incisor teeth of young rats killed 24 h after an intravenous injection of ^{45}Ca. The labeling is the same as in Figs. 1—5. (1-day exposure). Fig. 4. Low power view. The arrowhead points to the radioactive band of dentin, now located at some distance from the predentin-dentin junction. A fairly uniform reaction is observed over the enamel, $\times 100$. Fig. 5. Higher power view of the same preparation as in Fig. 4. Gradients of reaction extend from the radioactive band of dentin in both directions. The reaction observed over predentin and cells is slight. A fairly uniform reaction is seen over enamel, $\times 250$

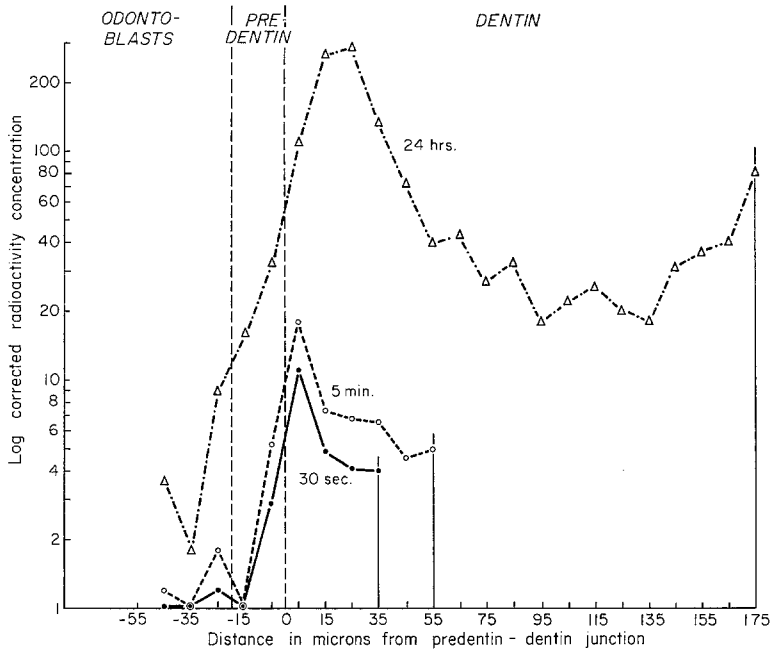


Fig. 6. Comparison of the grain counts (corrected for a 36-h exposure as explained in text) over odontoblasts, predentin and dentin of incisors of rats killed at 30 sec, 5 min and 24 h after injection of 10 μCi of ^{45}Ca/g of body weight. The ordinate shows the log of the corrected grain counts. The abscissa shows the origin (*O*) corresponding to the predentin-dentin junction (with a vertical broken line). At the right of the origin, the grain counts on dentin are presented up to the dentin-enamel border (which is represented by a vertical line for each animal). At the left of the origin, another broken line corresponds to the limit between predentin and odontoblasts

rat. In the case of this tooth, he measured calcium uptake either by calculating it from the amount of radioactive calcium taken up hourly over an 18-h period, or by dividing the ash weight of the tooth by the time required for its renewal, (45 days). Both methods gave a similar result. He concluded that all or nearly all the labeled calcium entering the tooth did so by an irreversible process and, therefore, was retained for the growth of the tooth. Bauer and Shtacher (1968) came to the same conclusion by comparing the rate of deposition of the mineral precursor, ^{85}Sr, to the rate of collagen synthesis measured by ^{14}C-proline uptake. The ratio ^{14}C:^{85}Sr was found to increase in bones over the first four days after injection, but to remain fairly constant in the incisors. The increase in the bone ratio was attributed to loss of ^{85}Sr by reverse exchange, whereas the constancy of the tooth ratio was explained by retention of the label. The authors concluded that, in the incisor tooth "the effect of mineral exchange was minimal or absent". Finally, our attempt at removing labile calcium from dentin by flooding the body with "cold" calcium ions following ^{45}Ca injection was ineffective (Table 4). For these various reasons, it is believed that all or nearly all the labeled calcium entering the incisor tooth is retained until attrition occurs 45 days later and, therefore, may be considered as being added for growth.

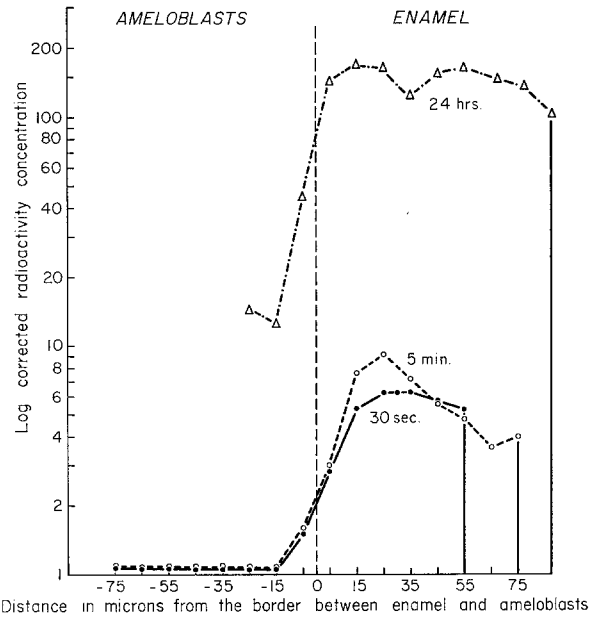


Fig. 7. Comparison of the grain counts (corrected for a 36-h exposure) over ameloblasts and enamel of incisors of rats killed at 30 seconds, 5 min and 24 h after injection of 10 μCi of ^{45}Ca/g body weight. The data were plotted as in Fig. 6, but the border between ameloblasts and enamel was taken as origin (*O*). The dentin-enamel border is represented by a vertical line for each animal

Role of the Cells of Dentin and Enamel in Mineral Deposition

The odontoblasts and ameloblasts respectively secrete the matrix of dentin and enamel and, in so doing, provide the material in which calcium phosphate is to be deposited. Whether the cells also play a direct role in the elaboration of the calcium phosphate is a controversial subject.

First, there was evidence that calcium is accumulated in odontoblasts (Kashiwa and Sigman, 1966; Fromme *et al.*, 1971) and ameloblasts (Kashiwa and Sigman, 1966), possibly as a preliminary step to its deposition into the matrix. Our examination of radioautographs immediately after injection of labeled calcium revealed that the grain counts were low over the cells (Tables 1 and 2) and dropped along an approximately straight line from dentin over predentin and odontoblasts (Fig. 6) and from enamel over ameloblasts (Fig. 7), suggesting that the decrease in the counts bore some relation to the distance from radioactive dentin or enamel. That the cell reactions were due to scatter of the β-rays emitted by the radioactive matrices was indicated by the finding that, when the odontoblasts were separated from dentin prior to radioautography, their grain count did not exceed background (Table 3). Hence, the odontoblasts—and probably the ameloblasts as well—did not contain radioautographically detectable labeled calcium.

If calcium were present in the cells (Kashiwa and Sigman, 1955; Fromme *et al.*, 1971), but not detectable in radioautographs after ^{45}Ca injection, the likely

explanation would be that the cell calcium was in a soluble form and, therefore, washed out in the course of processing. This soluble form might be the calcium ion. The cells would thus have the ability to concentrate this ion. Our results provide no evidence for or against this possibility.

The calcium found in some cells in the form of calcium phosphate (Lehninger, 1970; Martin and Matthews, 1970; Holtrop, 1971) was felt by these authors to be in the process of being "secreted" to the matrix. However, our results indicated that such calcium, if present in the cells of our animals, did not become significantly radioactive and, therefore, was not involved in the uptake of minerals for dentin and enamel. Such calcium might be involved in storage or other accessory phenomenon, but would not be an intermediate in calcification. It might be argued, however, that calcium phosphate is not completely insoluble and could be lost during processing. But if so, why would it be lost from the cells and retained in the matrix?

We are inclined to believe that if calcium is collected by the cells of hard tissues, it is in a readily soluble form, such as the calcium ion. The cells might pump calcium ions into mineralization sites and raise local concentration as a step toward calcification (Shapiro and Greenspan, 1969). We further believe that our observations do not support the opinion that calcium and phosphate combine within the cells of hard tissues and are then released as calcium phosphate to the matrix (as proposed by Watt in 1925, Pautard in 1966, Lehninger in 1970, Talmage in 1970, and Nichols and Rogers in 1971).

Initial Deposition of Calcium Phosphate in Matrices

In contrast to the lack of significant radioautographic reaction over the cells after ^{45}Ca injection, a strong reaction was observed over the matrix of dentin and enamel. A definitive pattern was observed as early as 30 sec (Fig. 2) and did not show significant change at 5 min (Fig. 3) or even at 1 h, as observed in less adequate radioautographs (Kumamoto and Leblond, 1956). Accordingly, the early pattern is a significant one. It is believed to demonstrate the sites of deposition of calcium phosphate in dentin and enamel matrix.

These observations support the view that calcium and phosphate combine at the sites of mineralization in dentin and enamel matrix (as proposed by Leriche and Policard in 1926, Ham in 1934, Leblond *et al.*, in 1950, Neuman and Neuman in 1953, and Glimcher and Krane in 1968).

2. Predominance of Dentin Radioactivity along the Predentin Border

Following ^{45}Ca injection, the radioautographic reaction consisted of a strong band along the predentin-dentin border, from which a decreasing gradient extended toward the dentin-enamel border (Figs. 2, 3). Since the dentin of the incisor tooth progressively thickens by accretion of new dentin on the inner surface (Schour and Massler, 1949), the band was over recently formed dentin, whereas the gradient was over a region of progressively older dentin as the enamel was approached. It was, therefore, likely that the calcium deposited next to the predentin-dentin junction contributed to the neoformation of calcium phosphate

crystals, whereas the calcium deposited deep into dentin served for the growth and completion of the crystals. Johansen and Parks (1962) have noted that, in human dentin, the calcium phosphate crystals are small next to the junction and large in the depth of dentin.

3. Interstitial Deposition of Radioactivity into Enamel Matrix

The radioautographs showed a fairly uniform interstitial deposition of labeled calcium in the enamel matrix, with some decrease next to the ameloblasts (which may be explained by the alternation of the non-mineralizing Tomes processes with the prongs of new mineralizing matrix). Preliminary observations in longitudinal sections (as far as they could be cut) also showed a fairly uniform distribution of radioactivity, except for a decrease where mineralization was advanced. These various observations were in fair agreement with previous radioautographic investigations after injection of ^{32}P-phosphate or ^{45}calcium (Bélanger, 1952, 1957; Leblond *et al.*, 1955; Kumamoto and Leblond, 1956; Reith and Cotty, 1962; Hammarstrom, 1967).

The appearance of label throughout the enamel matrix except in areas of advanced mineralization, is interpreted as indicating that the matrix starts accumulating calcium phosphate as soon as it is laid down and continues doing so at about the same rate until a stage of advanced mineralization is reached. Under such conditions, recently formed matrix should contain less mineral than the older one. Since the older the matrix is, the farther it is from secretory ameloblasts, the density of minerals should increase with the distance from these cells. Indeed, density measurements by means of birefringence (Crabb, 1959; Crabb and Darling, 1960) and microradiography (Engfeldt *et al.*, 1954; Engfeldt and Hammarlund-Esler, 1958; Crabb, 1959) revealed that the mineral content rose progressively as the distance from secretory ameloblasts increased.

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