Calcif Tissue Int (1990) 46:406–414

Calcified Tissue International

Changes in the Mode of Calcium and Phosphate Transport during Rat Incisal Enamel Formation

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Summary. The distribution of ^{45}Ca, $^{32}PO_4$, ^{22}Na, and calcein in the freeze-dried sections of rat lower incisor was examined. Also, the ratio of ^{45}Ca to $^{32}PO_4$ transported into the enamel at various developmental stages was studied after the simultaneous injection of ^{45}Ca and $^{32}PO_4$. The distribution of calcein fluorescence indicated the presence of an extracellular route from capillary to enamel in the areas of both the secretory and smooth-ended ameloblasts. Autoradiograms showed that the ^{45}Ca incorporation into the enamel in the smooth-ended ameloblast region was higher than that into the secretory enamel, and a remarkably high incorporation was observed in the enamel of the apical two-thirds of the ruffle-ended ameloblast region. Although the ^{32}P incorporation into the enamel of the smooth- and ruffle-ended ameloblast region was higher than in the secretory enamel, the differences between these two regions were not so evident as that observed in the case of ^{45}Ca. The high labeling of ^{45}Ca and ^{22}Na was observed in the apical two-thirds of the ruffle-ended ameloblasts. The $^{45}Ca/^{32}PO_4$ ratio in the secretory enamel was significantly lower than that in the blood, but in the enamel of the smooth-ended ameloblast region the ratio was not significantly lower. Contrarily, the ratio in the enamel of the ruffle-ended ameloblast region was much higher than that in blood. These results indicate that the mode of transport of these ions into enamel is altered in relation to the morphological changes of the ameloblasts.

Key words: Enamel formation — Autoradiography — Calcium — Phosphate — Sodium.

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It is well accepted that the mineralization of enamel proceeds in two phases involving the matrix-forming (secretory) and the maturation phase. In the secretory phase, the mineralization begins shortly after an organic matrix is secreted by the ameloblasts, and further mineralization proceeds rather slowly. After the entire thickness of enamel matrix is laid down, maturation phase takes place wherein there is a sudden and large influx of calcium and phosphate, and a marked increase of mineralization occurs. It is very important for a thorough understanding of the mechanisms of enamel formation to clarify the control system of calcium and phosphate transport by the enamel-forming cells and the pathway of these ions into enamel. Autoradiographic results [1, 2] showed that the calcium ion passed into developing enamel via the enamel organ, and the entry of the ion into enamel is controlled by the cells of enamel organ [3–5]. In order to define the control system and the pathway of calcium transport from the blood to the mineralizing enamel, the location of calcium in the enamel organ has been studied with histochemical methods [6–10], energy-dispersive X-ray spectrometry [9, 11–14] and autoradiography [3, 4, 14–18]. Also, the presence and localization of the Ca-ATPase activity in ameloblasts and the possible role of the enzyme in calcium transport has been intensively examined [19–24]. On the other hand, there is little information with regard to the phosphate transport [4, 25–27], and the findings vary. The difference between the calcium and phosphate control systems and their transport pathway through the enamel-forming cell layers are still inconclusive.

The object in the present study was to investigate the mode of ion transport and the pathway from the blood to the mineralizing enamel. In order to accomplish this, we examined (1) the possible existence of an extracellular route using calcein as a tracer; (2) the distribution of radioactive calcium,

phosphate, and sodium in the enamel-forming cells; and (3) the ratios of radioactive calcium to radioactive phosphate incorporated into the enamel at various developing stages.

Materials and Methods

Autoradiography

Autoradiographic procedures suitable for the study of water-soluble materials in whole-body sections, described previously by the authors [28], were used with some small modifications.

Nine-day-old rats of the Wistar strain (weighing about 20 g) anesthetized with ether were injected i.p. with 1% aqueous solution of calcein (0.1 ml/100 g body weight). Fifty minutes after the injection of calcein, 0.1 ml of solution of $^{45}CaCl_2$ (5 μCi/g body weight, NEN Research Products, USA), $H_3{}^{32}PO_4$ (1 μCi/g body weight, Japan Atomic Energy Research Institute), or $^{22}NaCl$ (1 μCi/g body weight, NEN) was injected i.p. After exposure to radioactive calcium for 3 minutes and to radioactive phosphate and radioactive sodium for 10 minutes, the anesthetized animals were rapidly frozen by immersion in cold hexane (−80°C). The frozen animal was embedded in 5% carboxymethyl cellulose solution (CMC), and was completely frozen in the coolant. The CMC block was attached to the stage of microtome (LKB 2258, Sweden), and was trimmed until a sectioned surface of interest appeared. The surface was covered with a sheet of Saran Wrap (Asahikasei Ind., Japan) coated with a synthetic rubber glue (Nenchakunori, Azia Gensi Co., Japan), and intimate contact between block and sheet was achieved by rubbing with a cotton wad. Sagittal sections (5–10 μm thick) of the lower incisor attached to the sheet were obtained and completely freeze-dried in the cryostat.

For contact autoradiography, the section surface was covered with Diafoil (plastic film of 4 μm thick, Mitsubishi Plastic Co., Japan) and contacted with X-ray film (Fugi Type 100, Fuji Photo Co., Japan). For microscopical autoradiography, the sections were covered with a dried thin film made of autoradiographic emulsion (Sakura NR-M2, Konica Co., Japan). After exposure, the X-ray film or the autoradiographic emulsion film attached to the section was developed with Rendol (Fugi Photo Co.) for 5 minutes at 20°C and fixed with Fuji Fix (Fuji Photo Co.) for 10 minutes. For the histological observation the section was stained with hematoxylin and eosin.

Measurement of Radioactivity at Various Stages of Developing Enamel

Nine-day-old rats were injected i.p. with 1% calcein solution (0.1 ml/100 g body weight) 1 minute prior to an i.p. injection of a mixture of $^{45}CaCl_2$ (100 μCi) and $H_3{}^{32}PO_4$ (100 μCi) solution. The animals were frozen in hexane (−80°C) 10 minutes after the injection of the radioisotopes, embedded with 5% CMC, and blocked in the coolant. Serial sagittal sections (15 μm thick) attached to a sheet of Saran Wrap were taken and completely freeze-dried as described above. Enamel samples of various developing stages, differentiated by the distribution of calcein fluorescence, were dissected with a razor blade from the lower incisor in the section, and the soft tissues were removed carefully from the surface of the enamel samples (Fig. 1). The width

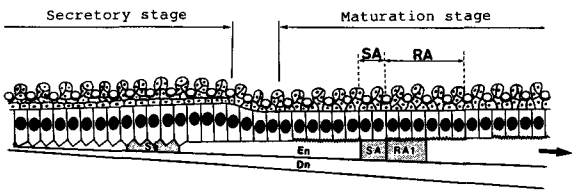

Fig. 1. Schematic drawings showing the position of sample dissected from the freeze-dried section of lower incisor of rat. SA = area of smooth-ended ameloblasts; RA = area of ruffle-ended ameloblasts; En = enamel; Dn = dentin.

of the sample was measured using a light microscope, and the area covered with ameloblasts in their respective developmental stage was estimated. The enamel samples of the same developmental stage were dissected from 15 serial sections and were placed in the vial containing a liquid scintillator (ACS-2, Amersham Co. Ltd, USA). The radioactivity was counted for 5 minutes every day during a period of 4 weeks. The radioactivity of ^{45}Ca and ^{32}P was estimated from their decay curves, respectively.

Results

Figure 2 shows a photomicrograph of the section of lower incisor prepared by the method described and stained with H-E for histological examination. Figure 3 shows the distribution of fluorescence on the adjacent serial section 10 minutes after an i.p. injection of calcein. In the secretory phase, the intensity of fluorescence in the papillary layer was much higher than that in the ameloblastic layer, and the calcein was incorporated into the enamel (Fig. 3A). Although the distribution of calcein in these two cell layers in the maturation phase was similar to that in the secretory phase, a relatively high fluorescence could be seen covering the whole thickness of the ameloblast layer in the middle part of the ruffle-ended ameloblast (RA) region (Fig. 3C). In some sections, the fluorescent band continued from the papillary layer adjacent to the RA to the enamel of the smooth-ended ameloblast (SA) region (Fig. 3B). The calcein was incorporated into the enamel of the SA region but not in the enamel of RA region. As is shown in the rectangular area in Figure 3B, which shows a cross section of ameloblast layer in the transitional region between the RA and SA, the fluorescence was located in the intercellular spaces.

Figure 4A shows the autoradiogram of the lower incisor of a rat sacrificed 3 minutes after an i.p. injection of $^{45}CaCl_2$. The radioactivity in the papillary layer in the secretory phase was much higher than that in the ameloblastic layer, and radioactive calcium was incorporated slightly into the enamel

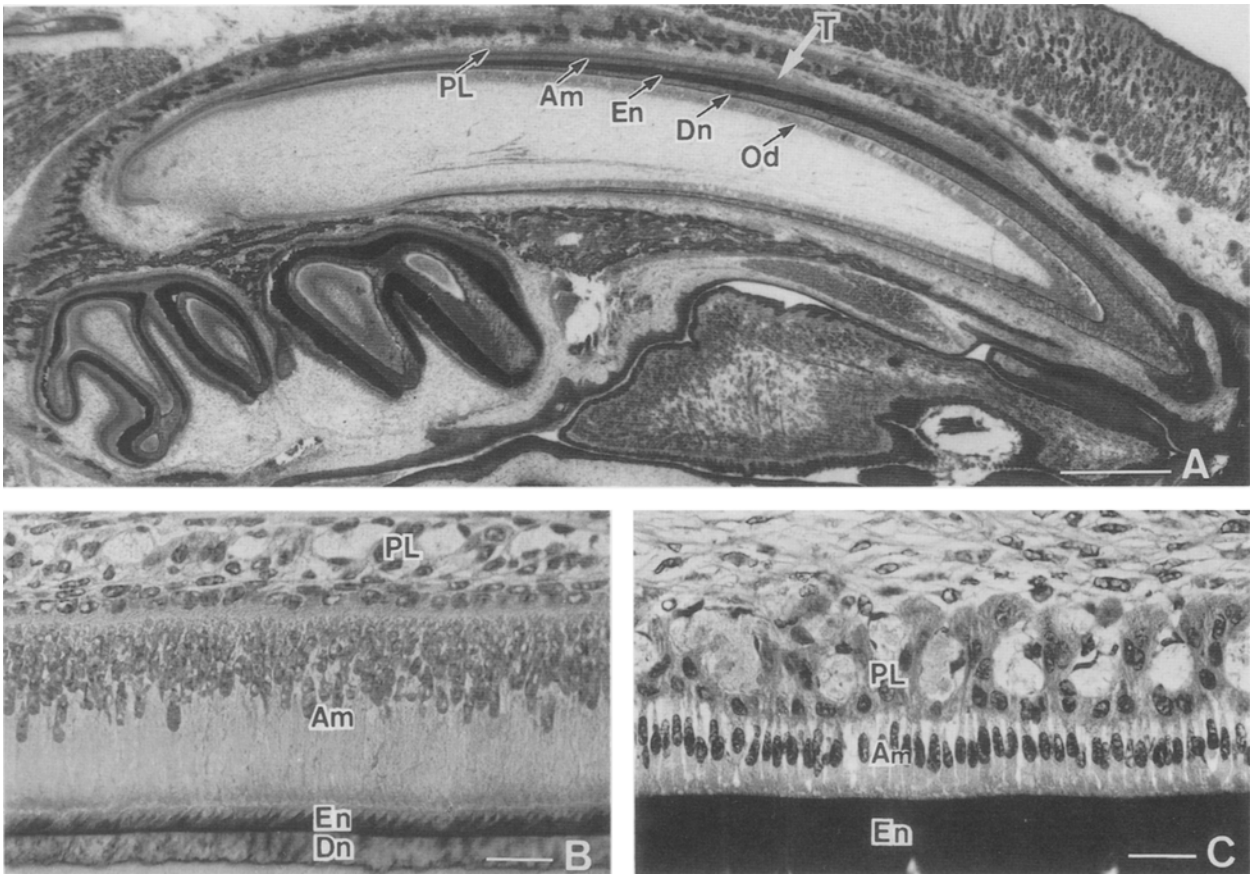

Fig. 2. Photomicrograph of the section of lower incisor of a 9-day-old rat stained with H-E (**A**). The area of the secretory and maturation stage, respectively, at a high magnification (**B, C**). PL = papillary layer; Am = ameloblast layer; En = enamel; Dn = dentin; Od = odontoblastic layer; T = transitional stage. The bar in (**A**) represents 1 mm, and the bar in (**B** and **C**) represents 30 μm.

(Fig. 4A). Although the radioactivity in the papillary layer in the maturation phase was high, the distribution of radioactivity either in the papillary layer or in the ameloblastic layer differed with the morphological changes of ameloblasts (Fig. 4A, B). Significantly high radioactivity was localized in the apical two-thirds of the RA region. In the enamel, radioactive calcium was incorporated into the area of the SA region and intensively into the area of the apical two-thirds of the RA region (Fig. 4B).

Figure 5 shows the autoradiogram of the lower incisor of a rat sacrificed 10 minutes after an i.p. injection of $H_3{}^{32}PO_4$. The radioactivity in the papillary layer was higher than that in the ameloblast layer in either secretory or maturation phase. The radioactive phosphate was incorporated into the enamel in the secretory phase, and the enamel of the SA region and of the apical two-thirds of the RA region in the maturation phase. Although in this case the highest uptake of radioactive phosphate into the enamel of the RA region was located in the middle part of the region, the location of the highest uptake varied within the limits of the apical two-thirds of the RA region.

Figure 6A shows the autoradiogram of the lower incisor of a rat sacrificed 10 minutes after an i.p. injection of $^{22}NaCl$. The autoradiogram showed a high labeling in the cell layers in the apical two-thirds of the RA zone. At a high magnification, it was observed that the labeling was located on the papillary layer and the basal side of the ameloblast layer (Fig. 6B). A high labeling around the capillary in the secretory ameloblast region is noticeable (arrows in Fig. 6C).

Table 1 shows the radioactivities and the ratios of radioactive calcium to radioactive phosphate in the enamel samples dissected from three developmental stages. The amount of either radioactive calcium or phosphate transported through a unit area (1000 μm^2) of ameloblast layer into the enamel of the secretory phase was low compared with those in other developmental stages. The amount of radioactive calcium transported through the unit area into the enamel of the SA region and the area of the

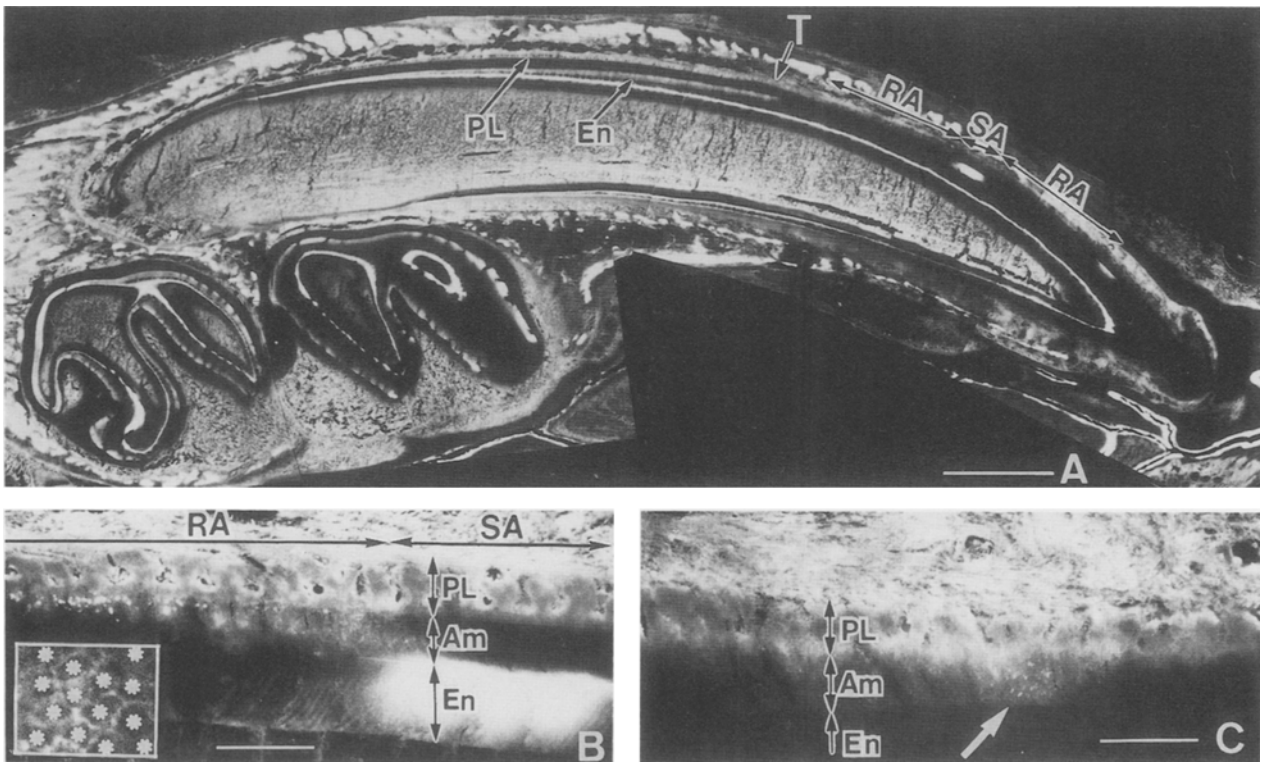

Fig. 3. Distribution of calcein fluorescence in the lower incisor of a 9-day-old rat 10 minutes after an i.p. injection (**A**). The transitional area from ruffle-ended ameloblasts (RA) to smooth-ended ameloblasts (SA) (**B**). The inset (**B**) shows the distribution of fluorescence in a cross section of ameloblasts in the transitional area from RA to SA. (*) indicates the ameloblasts, and an intensive fluorescence is distributed around the cells. The area of RA (**C**). The arrow in (**C**) indicates the portion where calcein is distributed to the whole thickness of the ameloblast layer. Abbreviations the same as in Figure 2. The bar in (**A**) represents 1 mm, and the bar in (**B** and **C**) represents 80 μm.

apical half of the RA region were about 2.2 and 4.9 times higher, respectively, than that in a comparable region of the secretory phase. The amount of radioactive phosphate transported through the unit area into the enamel of the SA or RA region was approximately 1.4 times higher than that in the secretory phase. The ratio of radioactive calcium to radioactive phosphate in the enamel of the secretory stage was lowest of all the developing stages and was lower than that of the blood. The ratio in the SA zone was slightly lower than that of the blood, but the ratio in an apical half of the RA zone was significantly higher than that of the blood.

Discussion

In our previous study [28], the exposed tissue surface was covered with a piece of photocopier paper and cellulose tape (Scotch tape 810), a section was made, and then the section was transferred onto a glass slide coated with synthetic rubber glue. However, in the present study we used a sheet of Saran Wrap coated with synthetic rubber glue instead of the combination of copier paper and cellulose tape. This modification made it easier to prepare thinner sections (5 μm) than the previous method; also the section attached to the Saran Wrap could be mounted on a glass slide for histological examination. As shown in Figure 2, the section of a 9-day-old rat lower incisor prepared by these procedures had no noticeable damage, and the tissues were preserved well enough for histological and autoradiographic examination at a high magnification (Fig. 2).

Although this method does not distinguish between the state of the incorporated calcium or phosphate into the enamel, e.g., free ions, adsorbed or exchanged ions, or those incorporated into hydroxyapatite crystals, it has the advantage of avoiding contact with aqueous fluid and is therefore likely to maintain water-soluble tracers *in situ*. For the purpose of the present study, which was to investigate the mode of ion transport in the secretory and maturation phase of amelogenesis and the ion pathway from the blood to the mineralizing enamel, the method is satisfactory if it is possible to reduce

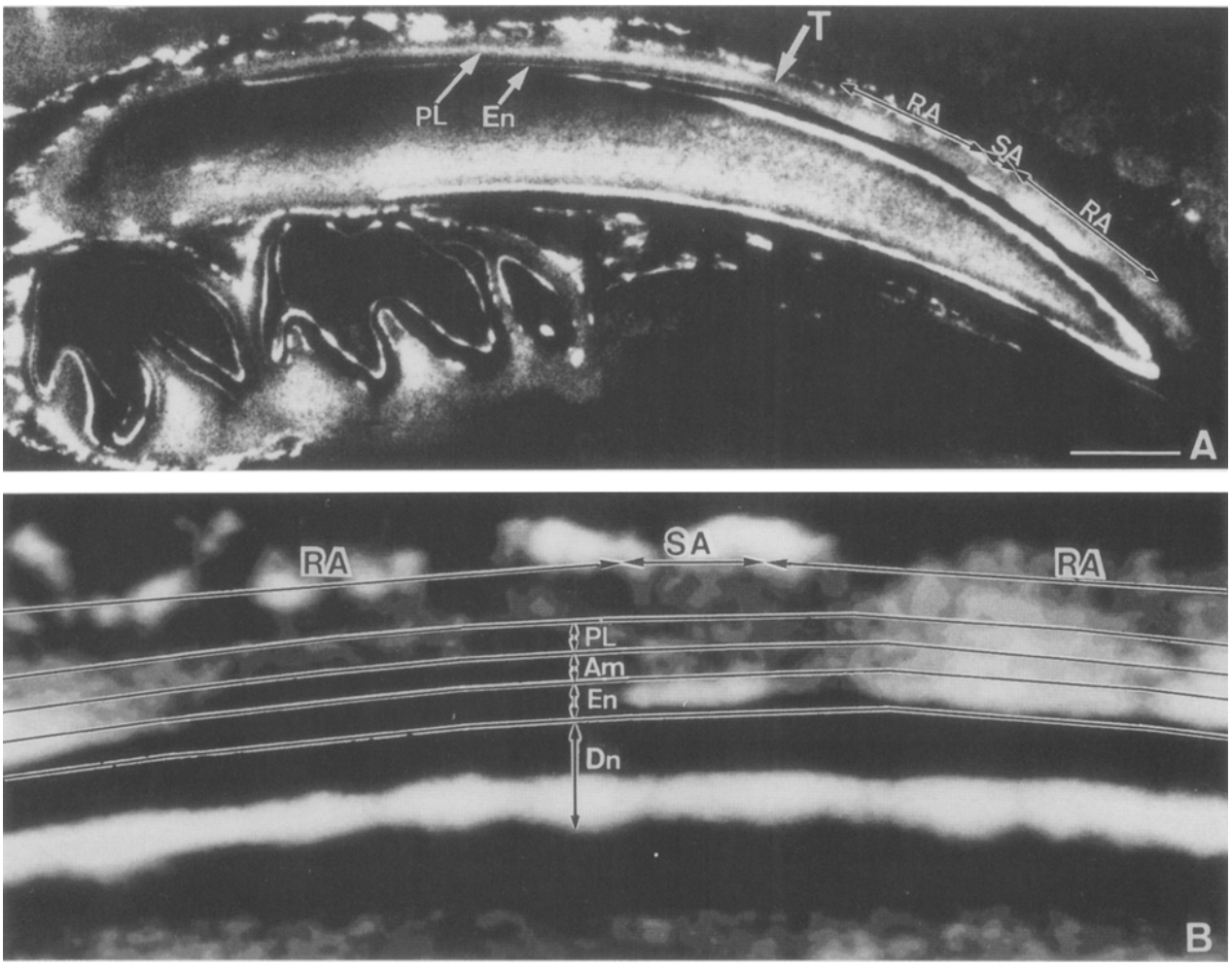

Fig. 4. Contact autoradiograph of the lower incisor of a 9-day-old rat 3 minutes after an i.p. injection of $^{45}CaCl_2$ **(A).** A graphic analyzer contour map representation of the autoradiogram in the maturation **(B).** White areas correspond to the high radioactive regions. Abbreviations same as in Figures 2 and 3. The bar represents 1 mm.

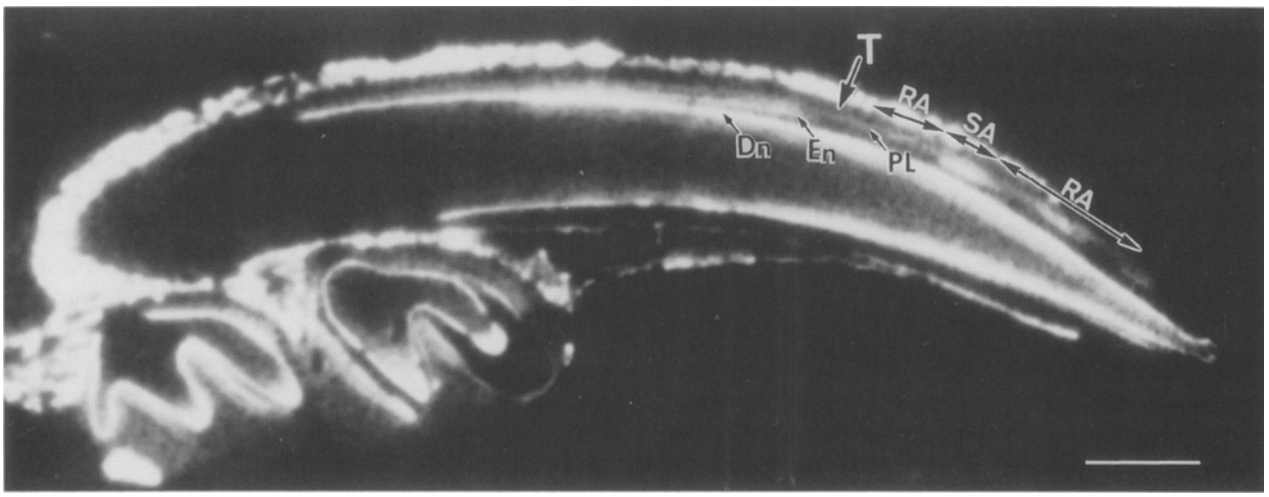

Fig. 5. Contact autoradiograph of the lower incisor of a 9-day-old rat 10 minutes after an i.p. injection of $H_3{}^{32}PO_4$. White areas correspond to the high radioactive regions. The bar represents 1 mm. Abbreviations same as in Figures 2 and 3.

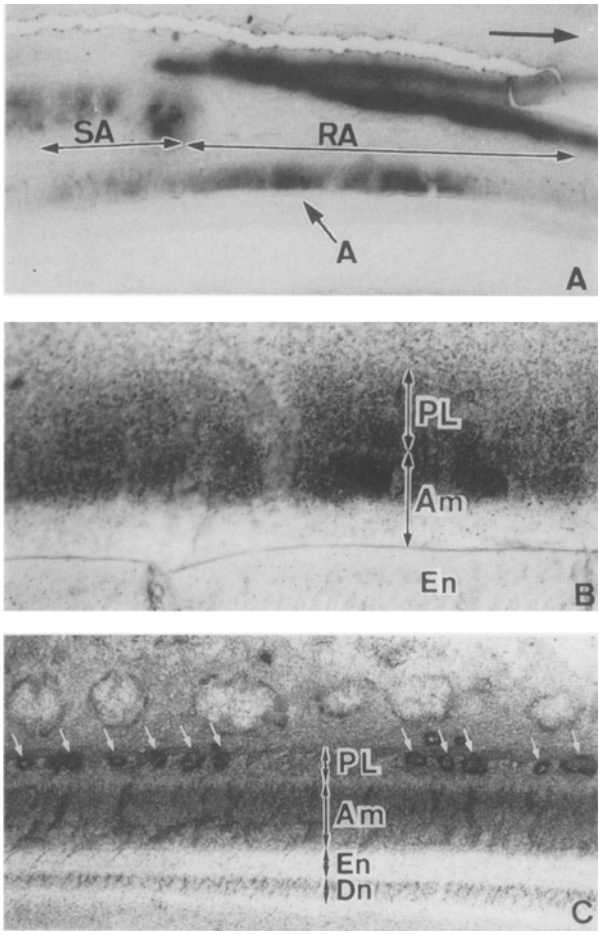

Fig. 6. Microscopical autoradiographs of the lower incisor of a 9-day-old rat 10 minutes after an i.p. injection of $^{22}NaCl$. The maturation stage **(A)**, and **(B)** the area indicated with an arrow A in Figure A at a high magnification. C shows the area of the secretory stage **(C)**. Arrows indicate the capillary. Abbreviations same as in Figures 2 and 3.

ion mobility in order to minimize the undesirable diffusion and ion exchange process between the blood and enamel-forming cell layers, and also between the cell layer and the enamel during the transportation of these ions into enamel. Therefore, in an attempt to achieve these objectives, and also to get adequate counts for the measurement of radioactivity in the enamel samples, we selected as a period of exposure time 3 or 10 minutes after the administration of the tracer.

It has been shown that the calcein is incorporated into the developing enamel wherein it is distributed to at least one, but usually several widely spaced bands in the position of the SA [29, 30]. These findings were also confirmed in this study. Furthermore, in the ameloblast layer the calcein fluorescence was localized in the extracellular spaces, as shown in Figure 3B. This would indicate that the calcein is a useful tracer to reveal the extracellular pathways from the blood to the enamel.

Figure 7 illustrates the summary of the distribution patterns of radioactive calcium and phosphate. Taking into account the distribution patterns of calcein in the developing enamel, one could speculate that (1) the radioactive calcium and phosphate can pass from the blood to the enamel through the extracellular spaces of the ameloblast layer in the secretory stage and between the cells of the SA layer; and (2) that these ions pass through the RA layer intracellularly.

Josephsen and Fejerskow [31] showed that the intercellular spaces between typical RA communicated freely with those of the papillary layer but were closed toward the enamel by the junctional complexes at their distal ends. On the other hand, the intercellular spaces between the SA were open to the enamel surface but closed towards the papillary layer. Takano and Ozawa [32, 33] showed that the distal junctions between the RA prevented horseradish peroxidase from reaching the enamel. Kallenbach [34] showed that although the SA and transitional ameloblast areas allowed the horseradish peroxidase to reach the enamel-ameloblast interface, the basal intercellular junctions between the SA appeared to form a barrier to the passage of the tracer. He also postulated the possibility that the tracer moved laterally from the adjacent transitional RA region to the SA region intercellularly. Figure 3B might show this roundabout pathway for the calcein, and indicates the presence of an intercellular route from the papillary layer to the enamel for calcium and phosphate in this area. The ratio of radioactive calcium to phosphate, which were incorporated in the enamel of the SA region, was not so different from the ratio in the blood. This would also indicate that most of the calcium and phosphate ions penetrate intercellularly without control by the smooth-ended ameloblast cell. On the other hand, in the RA region the intercellular route is closed, and most of the calcium and phosphate ions, especially calcium ions, are transported by the cells. This is clearly indicated by the high ratio of radioactive calcium to phosphate in the enamel of the RA region. Although at first Takano and Crenshaw [25, 35] described the absence of Ca-ATPase activity from the maturation ameloblasts and postulated that the maturation ameloblasts actively transported calcium by the Na-Ca exchange mechanism through the plasma membrane, later Takano et al. [23, 24] demonstrated that in the iodoacetoamide-treated specimens, intense Ca-ATPase activ-

Table 1. The radioactivities and ratios of ^{45}Ca and $^{32}PO_4$ in the enamel of the secretory and maturation stages

	Blood	Se	SA	RA1
Total width (μm)	—	34848	4257	5709
Total area (μm²)	—	522720	63855	85635
Total counts (cpm) (calcium)	3011 ± 25	738 ± 12	199 ± 6	590 ± 11
Total counts (cpm) (phosphate)	6638 ± 36	2771 ± 24	467 ± 10	660 ± 11
^{45}Ca (cpm)*	—	1.41 ± 0.02	3.12 ± 0.09	6.89 ± 0.13
$^{32}PO_4$ (cpm)*	—	5.30 ± 0.05	7.31 ± 0.02	7.71 ± 0.13
Ratio (Ca/P)	0.45 ± 0.01	0.27 ± 0.01	0.43 ± 0.02	0.89 ± 0.02

The positions of the sample dissected are indicated in Figure 1. ^{45}Ca* and $^{32}PO_4$* indicate the amount of radioactive calcium and phosphate transported through a unit area (1,000 μm²) of ameloblast layer into enamel. Counts are mean ± SD

ity was localized along the outer surface of ruffled distal membrane and in the adjacent tubulovesicular structures of the RA, whereas they were not shown along the lateral plasma membranes proximal to the distal junctional complexes. As shown in Figure 6, the distribution of radioactive sodium was localized on the cell layers in the apical two-thirds of the RA region and the labeling was located on the papillary layer and the basal side of the ameloblasts. Taking these results into consideration, one may speculate that in the RA the calcium is actively incorporated into the cells by the Na-Ca exchange mechanism and transported into the enamel by the Ca-ATPase system.

Most autoradiography of the developing teeth has shown a limited uptake of the radioactive calcium in the secretory enamel, and Bawden et al. [3, 4, 15] suggested that the secretory ameloblast layer acted as a barrier to restrict the flux of calcium to the enamel. Because intense Ca-ATPase activity was demonstrated along the outer leaflet of the almost entire plasma membrane except for the area of the tight junction of the secretory ameloblasts [19, 21, 22], Crenshaw and Takano [25] postulated that calcium is pumped out from the ameloblasts into the two different compartments being separated by the distal junctional complexes. Therefore, most of the calcium extruded from the ameloblasts is directed back to the papillary layer side and only a small amount of calcium is transported into the enamel. Our present result—that the calcein can reach the enamel through the extracellular spaces of the secretory ameloblast layer—could not exclude the possibility of an intercellular pathway for calcium and phosphate ions. However, the fact that the ratio of radioactive calcium to phosphate incorporated into the secretory enamel was lower than in the blood and other parts of the enamel would indicate that the entry of calcium ions was preferentially restricted by the secretory ameloblasts. The ameloblasts may push calcium ions to the papillary layer

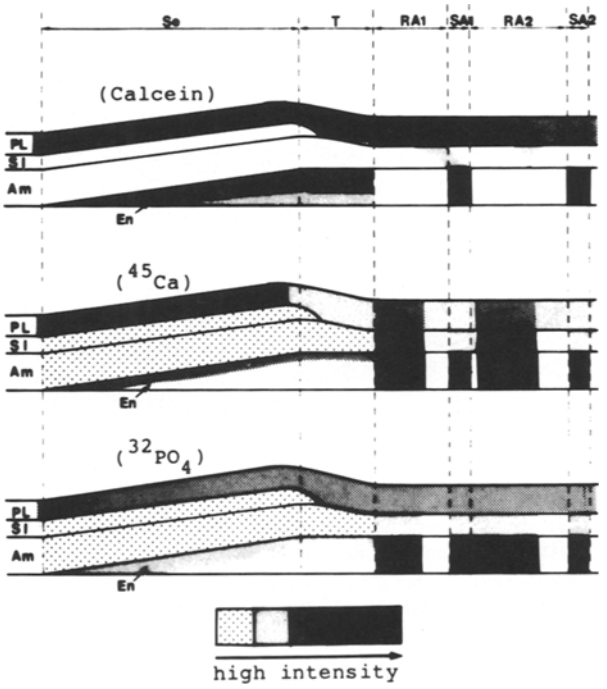

Fig. 7. Schematic drawing of the distribution of calcein, ^{45}Ca, and $^{32}PO_4$ in the enamel organ of the lower incisor of 7 to 9-day-old rats. Se = area of secretory stage; T = area of transitional stage; RA1, RA2 = area of ruffle-ended ameloblasts; SA1, SA2 = area of smooth-ended ameloblasts; PL = papillary layer; SI = stratum intermedium; Am = ameloblast layer; En = enamel.

by the action of Ca-ATPase. The relatively high uptake of radioactive sodium by the secretory ameloblasts might be related to the cell's activity transporting various components for the synthesis of the enamel matrices.

In a previous article [14] we reported a high uptake of radioactive calcium and phosphate by the papillary layer cells. In addition, the present study shows a high uptake of radioactive sodium by the cells, especially by the cells of the capillaries. This

would also indicate high transporting activities of these cells.

Although the precise mechanism, especially biochemical, of transport during the formation of enamel is still obscure, taking our present results into consideration, one might conclude and speculate the following: The ions and molecules related to the formation of enamel are actively and selectively transported from the blood to the ameloblasts by mutual cooperation of the capillary cell and the cells of the papillary layer. The secretory ameloblasts actively incorporate the components for the synthesis of enamel matrices, secrete the matrices, and extrude calcium to the capillary side to maintain the enamel at a low level of calcification. In the maturation stage, the ameloblasts control the entry of calcium and phosphate into the enamel by changing the tightness of intercellular junctions and/or their activities for ion transport.

Acknowledgments. We are indebted to Professor R. W. Fearnhead for his valuable comments and advice. This study was supported in part by Research Grant No. 62771509 and No. 63480417 from the Ministry of Education of Japan.

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Received June 5, 1989, revised August 7, 1989.