Biomineralization during early stages of the developing tooth *in vitro* with special reference to secretory stage of amelogenesis

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ABSTRACT In this survey we summarize data on mineralization of enamel mostly obtained in organ culture experiments in our laboratory. Historically, the enzyme alkaline phosphatase has been proposed to stimulate mineralization by supplying phosphate or by splitting away inorganic pyrophosphate PP_i, a potent inhibitor of mineralization. Localization of alkaline phosphatase in developing teeth by enzyme histochemistry shows that cells of the stratum intermedium contain extremely high levels of alkaline phosphatase but secretory ameloblasts that are engaged in deposition of the matrix and in transport of mineral ions lack alkaline phosphatase. The function therefore must be an indirect one, since no activity was seen at the site of enamel mineralization. We propose that the main function of alkaline phosphatase in the stratum intermedium is to transport phosphate or nutrients from blood vessels near the stratum intermedium into the enamel organ. Another function of the enzyme in stages of cell differentiation was deduced from inhibition experiments with the specific alkaline phosphatase inhibitor l- pBTM, showing that in tooth organ culture the enzyme may be involved in the generation of phosphorylated macromolecules from P ions originating from pyrophosphate. Calcium plays an indispensable role in enamel mineralization *in vitro*. Low calcium concentration in the culture medium prevented initial dentin mineralization and enamel formation. Moreover, differentiating ameloblasts did not become secretory, in contrast to odontoblasts that secreted a layer of predentin matrix. Variations in phosphate concentration in the culture medium do not seem to affect tooth organ cultures adversely during mineralization *in vitro*. Exposure to F^-, however, has adverse effects on enamel mineralization depending on concentration and exposure time and produces a variety of disturbances. Many of the fluoride-induced changes in the enamel organ are reversible: young ameloblasts recover and resume secretion and mineralization of the fluorotic matrix when fluoride is removed from the medium. This recovery is enhanced when medium calcium levels are increased. Only the changes in the hypermineralized enamel remain irreversible. Thus, we hypothesize that fluoride induces a local hypocalcemia in the enamel fluid surrounding the enamel crystals by stimulating a hypermineralization of the pre-existing enamel crystals.

KEY WORDS: *developing teeth, in vitro biomineralization, alkaline phosphatase, fluoride, calcium*

Introduction

The development and mineralization of teeth have been the subject of mostly descriptive or analytic investigations (Schroeder, 1991). However, few attempts have been made to establish how mineralization might be studied experimentally (Wigglesworth and Hayward, 1973; Rahkamo, 1974; Bawden and Wennberg, 1977) and how results emanating from different disciplines might be related to each other, principally because of the lack of a well-controlled model for manipulating enamel formation without interference from the homeostatic mechanism of the body. To quantify biochemically and histologically the direct relationships of specific components or processes, that might be relevant for the development and subsequent biomineralization of teeth without the interference of systemic factors such as calcium homeostasis, well-controlled organ culture conditions adapted for studying the *in vitro* development of the tooth germ are needed.

In the present study we review results obtained in our laboratory using an organ culture system that allows us to acquire and quantify normal enamel and dentin mineralization *in vitro* (Bronckers, 1981; Wöltgens *et al.*, 1982). Special attention is given to the early mineralization events in enamel, based on modulations of enamel development and mineralization during organ culture.

Abbreviations used in this paper: F^-, fluoride.

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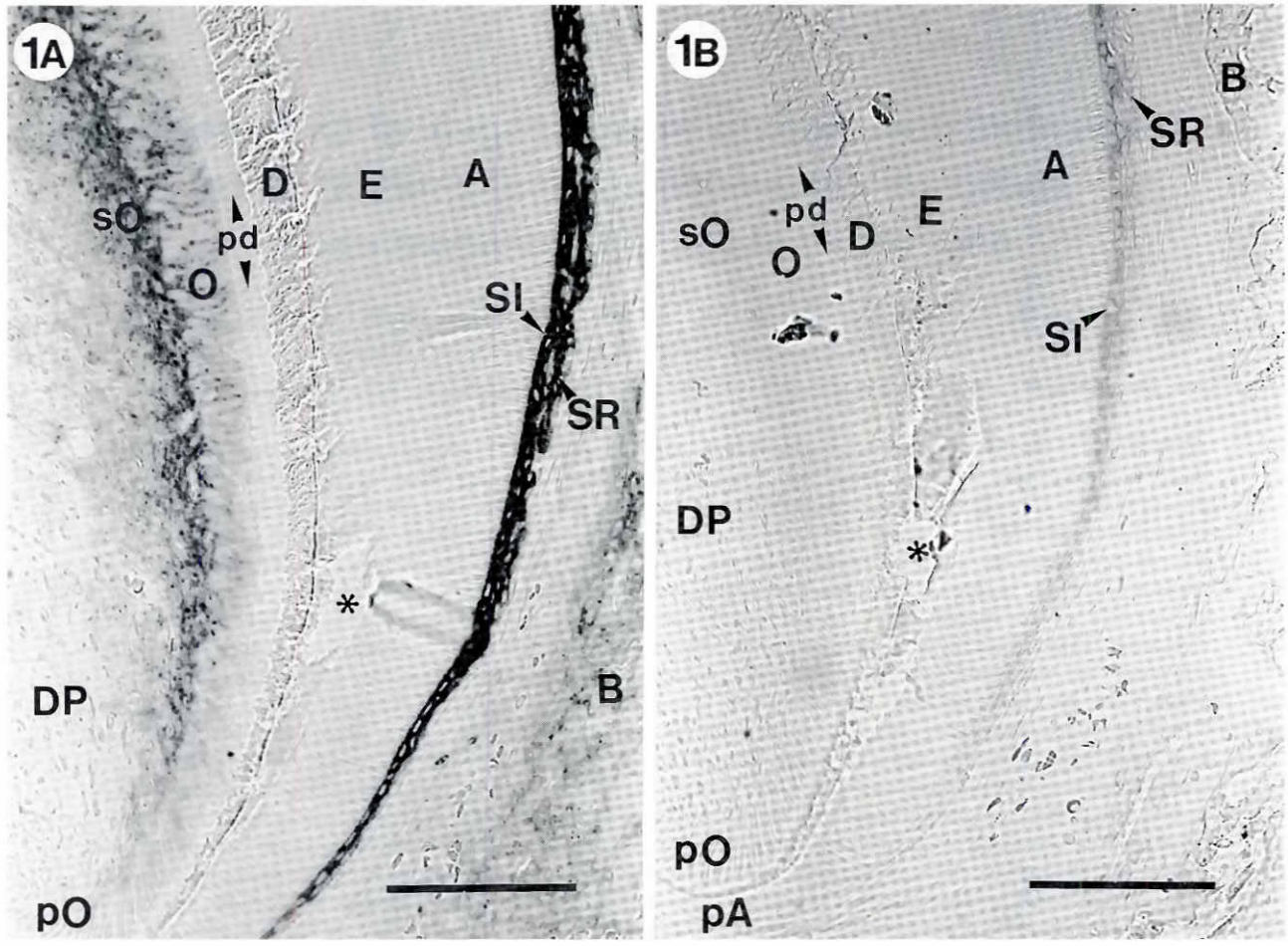

Fig. 1. Histochemical staining for alkaline phosphatase activity in a 4 day old developing hamster molar tooth germ, stained with naphtol-AS-TR phosphate and fast blue BB as substrate. (A) *Strong staining for alkaline phosphatase was revealed in the stratum intermedium (SI) and subodontoblastic (sO) layer adjacent to the secretory odontoblasts (O). In the odontoblasts the enzyme reaction is restricted to the lateral plasma membranes only. Apart from the low to moderate activity in the osteoblasts associated with the alveolar bone (B), the rest of the tissue appears negative for the enzyme reaction.* **(B)** *Control section, incubated in substrate solution containing 1 mM of parabromotetramisole (an inhibitor of non-specific alkaline phosphatase) exhibited only a weak staining over the stratum intermedium (SI). Historesin® (plastic) sections, undemineralized, not counterstained. Abbreviations: A, secretory ameloblasts; B, alveolar bone; D, dentine; DP, dental pulp; O, secretory odontoblasts; pA, preameloblasts; pd, predentine; pO, preodontoblasts; sO, subodontoblastic layer. * sectioning artefact. (Bar, 100 μm).*

We have restricted ourselves to findings mainly on a) the role of alkaline phosphatase in enamel mineralization, b) the modulation of secretion and biomineralization by calcium and phosphate regulation, and c) fluoride effects during the early stages of amelogenesis.

The role of alkaline phosphatase in enamel mineralization

High alkaline phosphatase (AP-ase EC 1.3.1.3) activity has been associated for a long time with the cells of mineralizing tissues such as bone (Robison, 1930) and teeth (Bonting and Nuki, 1963; Wöltgens *et al.*, 1970; Larmas and Thesleff, 1980). By virtue of its high activity in calcifying tissues, the enzyme, alkaline phosphatase has for many years been implicated in mineralization presumably as a supplier or generator of phosphate necessary for the induction and/or progression of the mineralization processes. Originally it was thought that the function of the enzyme in mineralizing tissues was to increase the local concentration of inorganic phosphate at the mineralization front by hydrolysis of hexose mono- and diphosphates to the point where calcium phosphate would spontaneously precipitate (Robison, 1930).

However this theory could not explain why extraskeletal tissues with high alkaline phosphatase activity do not mineralize and also could not account for the fact that the plasma level of these phosphate esters is too low to generate sufficient inorganic phosphate for spontaneous calcium phosphate precipitation. With this in mind, Neuman and Neuman (1958) proposed an alternative theory, suggesting that mineralization would occur only in tissues with templates (i.e., collagen) that facilitate precipitation of calcium phosphate salts. Fleisch (1964) showed that most tissues contain such templates but that these tissues are prevented from becoming mineralized because they also contain mineralization inhibitors. He also reported that phosphate esters are amongst the most potent inhibitors of mineralization. Even small concentrations of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and particularly inorganic pyrophosphate (PP_i) at levels as low as 10^{-5}-

10^{-7} M inhibited mineralization. Fleisch (1964) postulated that mineralization is only possible in tissues containing enzymes that are able to remove (hydrolyze) these inhibiting phosphate esters. Dulce (1960) reported that PP_i can be hydrolyzed by inorganic pyrophosphatase (PP_i-ase), an enzyme present in high activity in mineralizing cartilage. In bone, Eaton and Moss (1968) demonstrated an inorganic pyrophosphatase activity that was also part of the alkaline phosphatase; in mineralizing hamster molars, Wöltgens *et al.* (1970), Wöltgens and Bervoets (1978) provided strong evidence that the alkaline phosphatase enzyme may have PP_i-ase activity too. Since PP_i is also present in teeth (Bisaz *et al.*, 1968) a function of alkaline phosphatase might be to remove small amounts of PP_i and thus induce or promote dental mineralization.

In cartilage the enzyme is localized in matrix vesicles that are involved in initial stages of mineralization (Ali, 1976; Felix and Fleisch, 1976). Such vesicles were originally demonstrated by electron microscopy in mineralizing dentine by Bernard (1972). According to Slavkin (1975), matrix vesicles in predentin contain both alkaline phosphatase and hydroxy-apatite. Thus alkaline phosphatase can induce the precipitation of apatitic mineral inside the vesicle by increasing the phosphate concentration there (Ali, 1976) or by eliminating inhibitors of mineralization to such an extent that spontaneous mineral precipitation takes place even in the absence of any template (Felix and Fleisch, 1976).

In developing mouse molars, DePorter and TenCate (1976) suggested a close relationship between alkaline phosphatase and calcium transport during enamel formation. However, in studies with hamsters fed on a phosphate-deficient diet, Wöltgens (1971) showed that only during mineralization phosphate, and not calcium uptake coincides with the increase in alkaline phosphatase activity. This was later confirmed by *in vitro* data (Wöltgens *et al.*,1982). L-parabromotetramisole (l-pBTM) inhibits specifically both p-NPPase as well as PP_i-ase activity in homogenates of developing teeth (Wöltgens *et al.*, 1985), obviously in an uncompetitive way (van Belle, 1972). When added to tooth organ cultures this inhibitor had a more profound effect on the uptake of phosphate ions than of calcium ions during stages of mineralization (Lyaruu *et al.*, 1983). Consequently they proposed that in developing teeth the enzyme was related more to phosphate metabolism and/or transport than with calcium, contrary to the suggestion made by DePorter and TenCate (1976).

With the aid of microdissection techniques, Bonting and Nuki (1963) were able to analyze and quantify biochemically the content of alkaline phosphatase in various dissected layers of the enamel organ and dental papilla of the developing hamster tooth germ. They found high alkaline phosphatase activity in stratum intermedium and subodontoblastic layer as opposed to low activity in ameloblasts and odontoblasts in mineralizing molars (Fig. 1). Recently, alkaline phosphatase mRNA signals have also been revealed in the cells of the stratum intermedium in rat molars by *in situ* hybridization (Helder, personal communication) (Fig. 2), indicating that these cells synthesize the enzyme.

Though evidence has been presented that alkaline phosphatase and PP_i-ase are identical (Wöltgens *et al.*, 1970), the PP_i-ase requires Zn^{2+} for its hydrolytic activity but the alkaline phosphatase does not when determined in tooth homogenates with the non-physiological substrate paranitrophenylphosphate (p-NPP). Moreover PP_i-ase activity (but not alkaline phosphatase) can be inhibited by cadmium ions (Cd^{2+}), perhaps by replacing Zn^{2+} from the active site of the enzyme molecule. Ascorbic acid is also able to inhibit selectively the PP_i-ase activity (Wöltgens *et al.*, 1971), which can

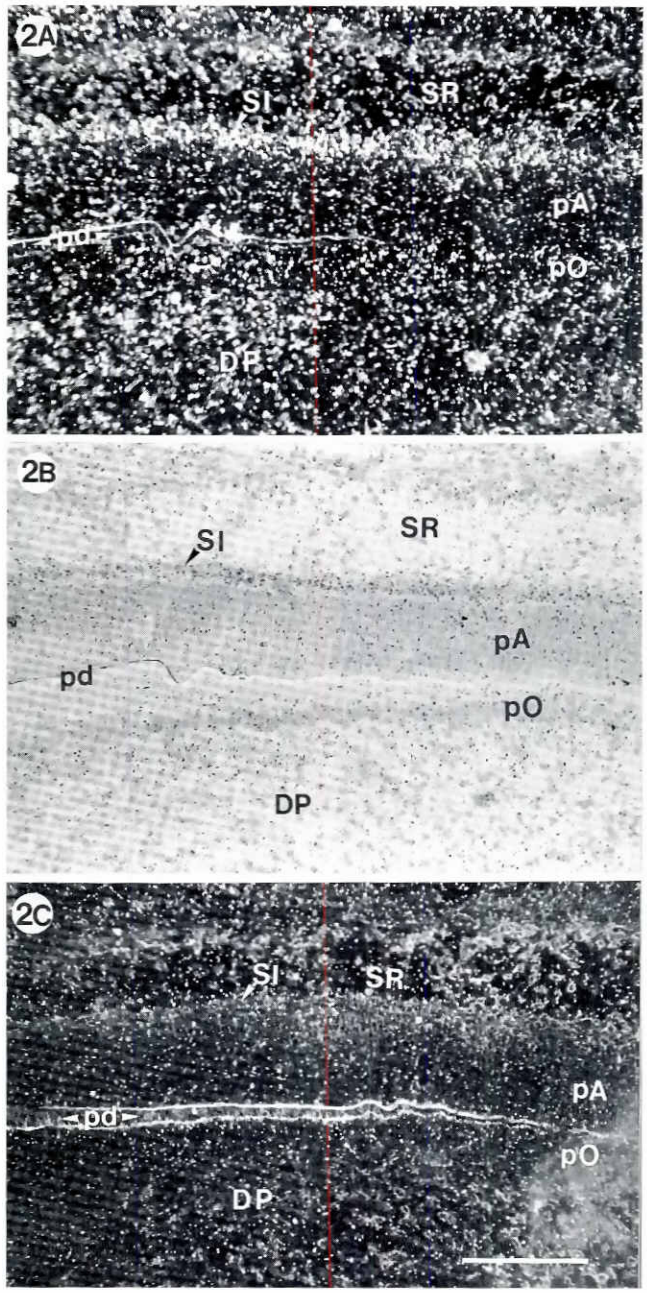

Fig. 2. Expression of alkaline phosphatase mRNA in a developing rat molar tooth germ shown by *in situ* hybridization. *Unfixed frozen sections from a 2 day old developing rat molar were incubated with ^{35}S-labeled riboprobes specific for rat bone alkaline phosphatase (a gift of Dr.G. Rodan, Merck, Sharpe and Dohm, USA). The sections incubated with antisense riboprobe* **(A)** *darkfield illumination;* **(B)** *bright field illumination showed a strong signal over the stratum intermedium (SI) and a diffuse signal over the stellate reticulum (SR) and dental pulp (DP). These signals were not seen in control sections incubated with sense riboprobe* **(C)**. **(A and B)** *Counterstained with hematoxylin. (Courtesy: Dr. M.H. Helder). (Bar, 100 μm).*

be reversed by addition of Zn^{2+} (Wöltgens, 1974). Apparently L-ascorbic acid removes Zn^{2+} from the active center of the PP_i-ase. For mouse tooth germs in culture, Togari *et al.* (1993) showed that addition of 100 μMol Zn^{2+} to the medium also increased the specific

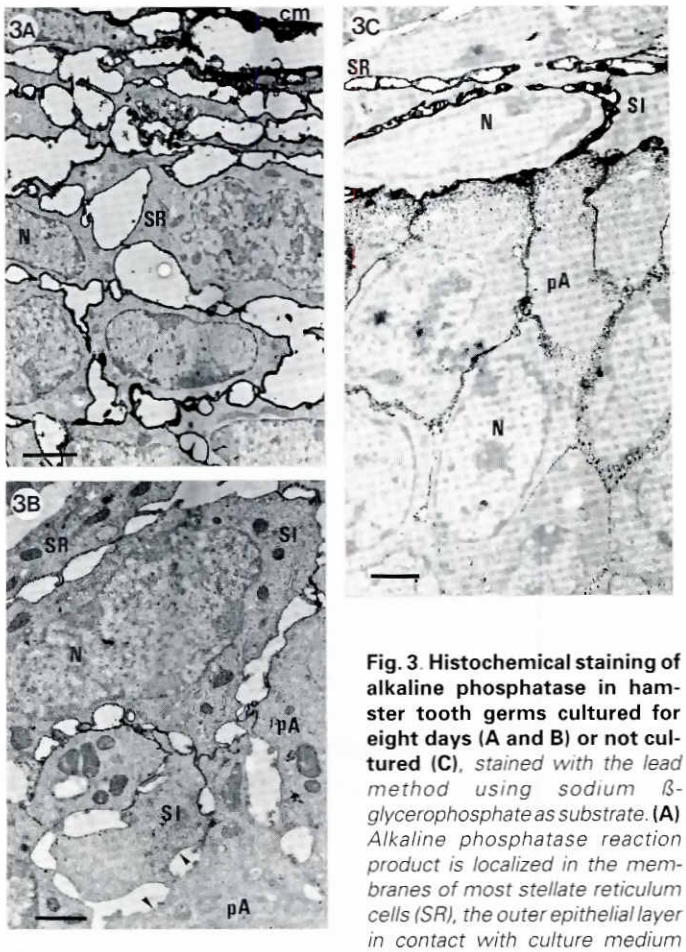

Fig. 3. Histochemical staining of alkaline phosphatase in hamster tooth germs cultured for eight days (A and B) or not cultured (C), *stained with the lead method using sodium ß-glycerophosphate as substrate.* **(A)** *Alkaline phosphatase reaction product is localized in the membranes of most stellate reticulum cells (SR), the outer epithelial layer in contact with culture medium (CM,* **A**) *and the stratum intermedium cells (SI) adjacent the preameloblasts (pA,* **B**). *Some reaction product is also seen in the membranes at the basal portions of the preameloblasts in contact with the stratum intermedium cells* **(B)**. **(C)** *In vivo, an intense reaction product is present over the membranes of the stratum intermedium cells (SI) and basal and lateral membranes of adjacent preameloblast (pA). In contrast to the cultured explants, the membranes of the stellate reticulum cells (SI) exhibited a faint staining at the level of the stratum intermedium (SI) that decreased towards the outer enamel epithelium. Abbreviations: N, nucleus. (Bars, A: 3 µm; B and C, 2 µm).*

activity of alkaline phosphatase, even if its activity is determined using the non-physiological substrate p-NPP. Apparently Zn^{2+} is not only required to activate the PP_i-ase activity as found in experiments on dental tissue homogenates, but may also stimulate the *de novo* synthesis of alkaline phosphatase protein, increasing thereby its specific activity even if determined with p-NPP as substrate.

In developing tooth germs *in vivo*, maximum alkaline phosphatase activity is always associated with the cells of the stratum intermedium (Bonting and Nuki, 1963). *In vitro* however, a shift of this high activity from the stratum intermedium cells to the stellate reticulum cells and cells of the outer enamel epithelium in contact with the culture medium has been observed (Fig. 3A-C; Lyaruu, 1983). These findings suggest a role of alkaline phosphatase in the transport and uptake of nutrients necessary for the enamel organ. The fact that *in vivo* in ameloblasts the enzyme is only present in the basal membranes facing the stratum intermedium cells and in odontoblasts only in the lateral plasma membranes, all remote from the mineralization front, makes it likely that the function of the alkaline phosphatase in mineralization is indirect by regulating transport of nutrients and/or of ionic transport. In this respect, the inhibition of the mineralization of newly formed enamel and dentine matrices by the specific alkaline phosphatase inhibitor l-pBTM *in vitro* could be indirectly the consequence of the suppression of the uptake of P (Lyaruu, 1983).

Furthermore Lyaruu *et al.* (1987b) showed that when $^{32}PP_i$ was added to the culture medium instead of $^{32}P_i$, phosphorylation of organic compounds was increased, especially in young non-mineralizing molars. This effect was also completely inhibited by the alkaline phosphatase inhibitor l-pBTM. Thus, the enzyme may also be involved in phosphorylation of organic macromolecules necessary for other activities related to cell differentiation, as recently suggested for alkaline phosphatase transfected cell lines (Hui *et al.*, 1993) and for levamisole-treated osteoprogenitor cells (Klein *et al.*, 1993). Apart from the physiological substrate PP_i, this phosphorylation also needs the cofactor Zn^{2+}, because replacement of the cofactor by Cd^{2+} results not only in the aforementioned inhibition of PP_i-ase activity (Wöltgens *et al.*, 1989a), but also in tooth organ cultures in an inhibition of phosphorylation of macromolecules (Wöltgens *et al.*, 1991).

Alkaline phosphatase from hamster tooth germs has been partially purified by high pressure chromatography and chromatofocusing or electrophoretically by Dogterom *et al.* (1984). Its molecular weight ranges from 50.2 kDa in the hamster (Dogterom *et al.*, 1984) to 16 kDa in the rat (Tojyo *et al.*, 1981;Tojyo, 1983). In the hamster its isoelectric point is 3.7 and no differences could be found between the alkaline phosphatase derived from the mesenchymal and epithelial part of the developing tooth (Dogterom *et al.*, 1984). For bovine dental tissues Oida *et al.* (1985) purified the enzyme up to 703 U/mg protein. The tooth-derived purified enzyme showed the same properties as the kidney-type isoenzyme and contained carbohydrate moieties, which react with concavalin A. They identified the enzyme as a dimeric glycoprotein consisting of two identical subunits each having a molecular weight of 80 kDa.

Collectively, from the *in vitro* data it can be derived that in developing teeth alkaline phosphatase stimulates mineralization indirectly by supplying phosphate or eliminating inhibitors of mineralization present in the extracellular tissue fluid such as PP_i. In the presence of PP_i the enzyme is also able to phosphorylate macromolecules and is involved in cell differentiation.

Modulation of secretion and biomineralization by calcium and phosphate regulation

The mineralization process in enamel differs greatly from dentin and bone not only morphologically but also biochemically (Butler, 1992). Glick (1979) and Engel (1981) reported calcium and phosphorus gradients in forming enamel in rat and mouse, which was confirmed by Stratmann *et al.* (1991) for mineralizing hamster enamel. They also observed qualitatively similar gradients of calcium and phosphorus in enamel deposited in organ culture. All data indicate that the concentration of both elements increase from enamel surface toward dentin-enamel junction as well as from the cervical loop toward the cusp tip.

With respect to the regulation of dentin and enamel mineralization by calcium and phosphate ions, Ferguson and Hartles (1966) reported histological and microradiographic changes in minerali-

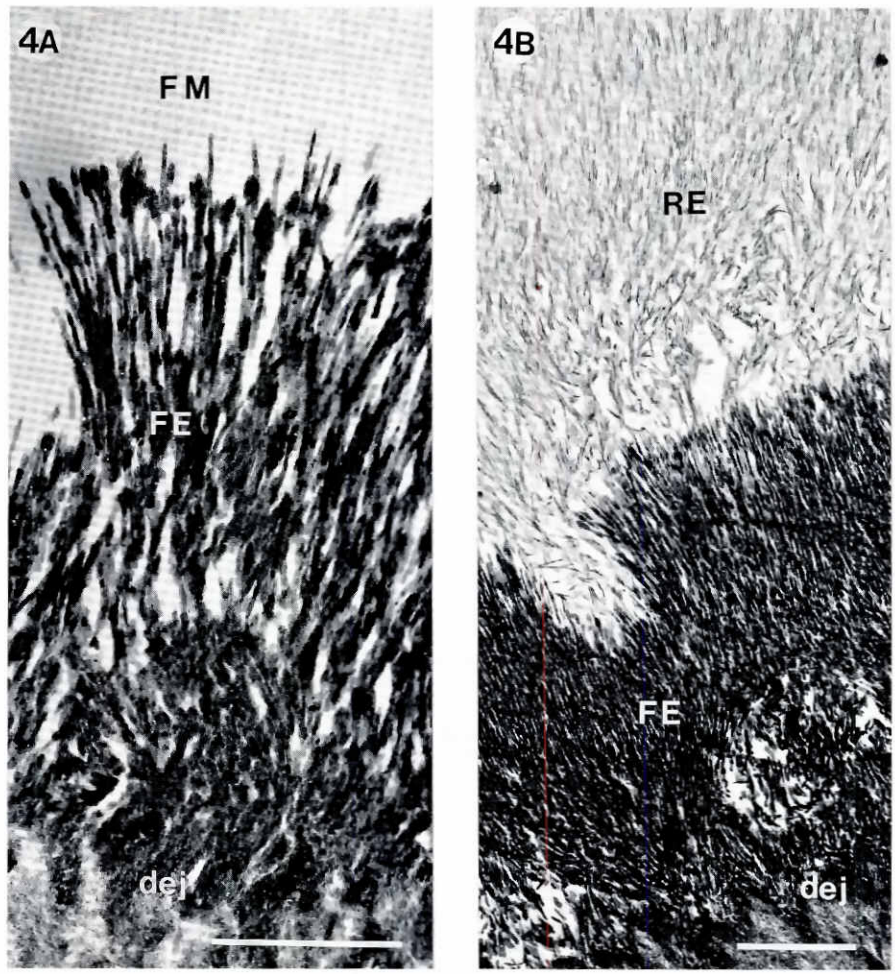

Fig. 4. Effect of fluoride on early enamel mineralization *in vitro*. **(A)** *Depicts an electron micrograph of early developing enamel from a hamster tooth germ explanted during the early stages of amelogenesis and then exposed to 10 ppm F⁻ for 24 h in organ culture and* **(B)** *shows a similar micrograph from a tooth germ also exposed to the same fluoride regime in vitro but followed by a further one day recovery culture period in a medium without the drug. Fluoride treatment* **(A)** *resulted in hypermineralization of the pre-exposure enamel (FE) deposited in vivo before the onset of culture and the secretion of unmineralized enamel matrix (FM). Enamel hypermineralization manifests itself as crystals having a high intrinsic electron density throughout the enamel layer – up to the dentin-enamel junction (dej) due to the high mineral content as a consequence of the fluoride treatment (see Lyaruu, et al. 1989a, for more details). The unmineralized fluorotic enamel matrix secreted during the fluoride exposure (***A****, FM) subsequently mineralized during the recovery culture period (***B****, RE). The fluoride-induced hypermineralization of the pre-exposure enamel was not affected by culture in fluoride-free medium (compare FE, Fig. A and FE,* **B**). *The recovery enamel (RE) layer deposited on top of the hypermineralized fluorotic enamel (RE) layer is comparable in structure and mineral content to that observed in control, untreated tooth germs (not shown; see also Lyaruu et al., 1989a,b, for more detailed data). (Bars, 1 μm).*

zation in incisors of rats maintained on diets deficient in calcium (Ca), phosphate or phosphate and vitamin D. Only the Ca-deficient diets significantly reduced tooth dry weight. Recently, Lozupone and Favia (1994) demonstrated that administration of low calcium diet to pups nursed by a mother also on a calcium-deficient diet, inhibited tooth growth and led to a dramatic but reversible reduction of mineralization of enamel without any effect on the amount of organic matrix deposition. Pindborg (1982) related enamel disturbances, known as enamel hypoplasia with neonatal hypocalcemia. Nikiforuk and Fraser (1979) studied patients with congenitally low plasma levels of calcium or phosphate and noticed that enamel formation was seriously affected when plasma calcium (but not plasma phosphate) is low during enamel development. On the other hand, when plasma phosphate is low, formation of dentin (and bone) is mainly affected. They suggested that for normal enamel formation, adequate plasma calcium levels are crucial. It remains unknown however whether plasma calcium levels act on enamel formation indirectly via the action of calcium homeostatic hormones or directly.

Wöltgens *et al.* (1987) presented *in vitro* evidence that amelogenesis is indeed considerably more sensitive to changes in calcium levels than in phosphate levels. In organ culture experiments using hamster molars they observed that lowering of the medium calcium concentration from 2.4 to 0.9 mMol prevented initial dentin mineralization and enamel matrix secretion but not secretion of collagenous predentin matrix. This phenomenon indicates that the secretion of enamel matrix is more sensitive to low levels of calcium than secretion of collagenous predentin although predentin failed to mineralize under low calcium conditions. Another possibility is that enamel matrix secretion and mineralization is triggered by the presence of a mineralized mantle dentin layer opposite the ameloblasts. Recently, these calcium-dependent changes in phenotypic expression have been associated with the presence of an extra vitamin D-dependent Ca-binding protein (Berdal *et al.*, 1993) and with a change in Ca^{2+}-ATPase (Takano *et al.*, 1986; Borke *et al.*, 1993) in secretory ameloblasts or with the protein kinase C (PKC)-related transduction system in young ameloblasts and odontoblasts during early stages of mineralization (Bawden *et al.*, 1994). Inversely, inhibition of enamel matrix secretion by microtubular inhibitors colchicine (Bronckers *et al.*, 1988) and vincristine (Lyaruu *et al.*, 1995) *in vitro* is also accompanied by a comparable decrease in calcium uptake.

Transepithelial transport of calcium ions toward the enamel mineralization front was examined by localization of ionic calcium in early stages of formation of dentin and enamel in tooth germ cultures using the K-pyroantimonate cytochemical technique (Lyaruu *et al.*, 1987a). These studies suggested that both odontoblasts and (pre)-ameloblasts are involved in the calcium

TABLE 1

SURVEY OF THE LOWEST F^- CONCENTRATION AFFECTING VARIOUS STAGES OF ENAMEL DEVELOPMENT *IN VITRO*

STAGE OF DEVELOPMENT	F^- TREATMENT			
	short term 1-2 days	parameter	long term 4-8 days	parameter
pre-ameloblasts	1.3 mMol	histology	1.3 mMol	histology
presecretory and early secretory ameloblasts	52 µMol	histology	1-2 µMol	biochemistry
secretory ameloblasts	52 µMol	biochemistry mineralization matrix breakdown	52 µMol	biochemistry mineralization matrix breakdown

acquisition necessary for the initial stages of mantle dentin mineralization, similar to *in vivo*. Indeed, during the initial stages of mantle dentin mineralization, there was also in the adjacent ameloblast an intracellular calcium gradient increasing to the mineralization front. At later stages of secretory amelogenesis, ionic calcium was located in the inner aspects of the secretory ameloblast membranes, decreased in the membranes of Tomes' processes and was visualized extracellularly at the enamel crystal growth points. These results supplement earlier *in vivo* and *in vitro* data indicating that the movement of ions including calcium into the developing enamel is controlled by the cells of the enamel organ (Bawden and Wennberg, 1977; Bawden *et al.*, 1982). The fact that during all stages of dentin and enamel mineralization most of the calcium in the (pre)-ameloblasts was accumulated in the apical poles of the cells suggests that there is an active calcium gradient in the direction of the mineralization front. This calcium gradient is probably perpetuated by the activity of Ca^{2+}-ATPase present in the extracellular side of the lateral plasma membranes of the secretory ameloblasts *in vivo* (Takano *et al.*, 1986; Takano, 1992; Borke *et al.*, 1993). The localization of calcium along the lateral plasma membrane of the secretory ameloblasts, as reported in these investigations, supports the transcellular calcium transport mechanism according to the model proposed by Reith (1983) and Reith and Boyde (1985). In agreement with data obtained from the non-cultured developing rat tooth germ (Deporter, 1977; Kogaya and Furuhashi, 1986) intracellular enamel matrix does not seem to be directly involved in calcium sequestration or its translocation to the enamel mineralization front. This opinion is further substantiated by autoradiographic studies using tritiated amino acids and ^{45}Ca, showing that matrix synthesis and secretion require more than 10 min, while calcium translocation from the circulatory system to the enamel mineralization front takes less than 30 sec (Leblond and Warshawsky, 1979). The Tomes' processes seem to regulate transmembrane calcium transport to the mineralization front. In areas where Tomes' processes did not develop, the enamel was devoid of the characteristic rod/interrod enamel prism arrangement and became completely aprismatic. Apparently the appearance of Tomes' processes is related with the rod/interrod arrangement of enamel prisms (Lyaruu *et al.*, 1987a).

Unlike calcium, phosphate uptake is regulated by alkaline phosphatase and seems to have – as mentioned earlier – only an indirect function in dental mineralization (Lyaruu, 1983). Contrary to the findings of Ameloot *et al.* (1987) high levels of P (3.6 mMol instead of 1.6 mMol) in the culture medium did not affect biochemical parameters such as ^{45}Ca and ^{32}P-uptake in mineralizing tooth organ cultures (Wöltgens *et al.*, 1987). Yet histologically, high phosphate medium levels resulted in the formation of a more regular but thinner enamel layer. High phosphate medium levels however were not able to induce *de novo* matrix secretion and mineralization in the enamel when medium calcium levels were low (Wöltgens *et al.*, 1987).

In short, from the *in vitro* data on the role of calcium in developing teeth obtained so far we may conclude that enamel formation and secretory ameloblasts are more sensitive to calcium depletion than odontoblasts in the process of dentinogenesis. Changing phosphate concentration in the organ culture medium *per se* will have only minor effects on amelogenesis in our *in vitro* system.

Fluoride effects during early stages of amelogenesis

It is well established that optimal fluoride concentration (1 ppm) in the drinking water will diminish dental caries drastically, but on the other hand that higher concentrations can lead to adverse effects on enamel formation resulting in mottled enamel (Ericsson, 1977). One reason for the sensitivity of forming enamel to F^- could be the affinity of fluoride for this tissue. Fluoride concentrations as high as 250 µg F^-/g dry weight have been found in developing enamel from various species obtained from non-fluoridated areas by chemical analysis of dissected enamel particles (Speirs, 1978; Weatherell *et al.*, 1975; Deutsch and Gedalia, 1982). Also the enamel organ soft tissue contains high fluoride concentration (Bawden *et al.*, 1992) along with a high calcium content. Remarkably, high fluoride content in forming enamel is mainly associated with the secretory phase of amelogenesis, but not with the more mineralized post- secretory stages. During the maturation stages the fluoride content in the developing enamel begins to decrease and reaches very low levels in the mature pre-eruptive enamel (Weatherell *et al.*, 1975; Speirs, 1978).

TABLE 2

REGULATION OF ENAMEL MINERALIZATION BY Ca AND F

medium Ca (mMol)	medium F (mMol)	enamel fluid between Tp* and mf** Ca (mMol)	transition from pre-ameloblast to secretory ameloblast	new matrix mineralization (OCP formation)	hypermineralization of pre-exposure enamel (HA formation)
2.1	-	$=10^{-2}$***	+	+	-
2.1	0.1	$\leq 10^{-2}$	-	-	+
4.2	-	$\geq 10^{-2}$	++	++	-
4.2	0.1	$=10^{-2}$	+	+	+

* Tp, Tomes process; ** mf, mineralization front; *** Aoba and Moreno (1987).

Studies on the distribution of fluoride injected into hamsters in early stages of secretory amelogenesis and measured by micro-Proton-Induced Gamma-ray Emission (PIGE) technique reveal that the highest levels of fluoride are incorporated in the area of the mineralizing mantle dentin. The entire area of secretory stage (pre-exposure) enamel also incorporates fluoride ions but at higher levels in the more mature enamel near the dentin-enamel junction than in the more superficial layers (Lyaruu *et al.*, 1989a; Tros *et al.*, 1993). *In vivo* enamel matrix secreted during exposure to fluoride fails to mineralize and forms a radiographic hypomineralized layer (e.g. Kruger, 1968, 1970; Walton and Eisenmann 1974). One may argue that this fluorotic matrix has different properties with regard to crystal formation than does the normal matrix. However organ culture studies of Bronckers and Wöltgens (1985) and recovery experiments of Lyaruu *et al.* (1987c) suggested that the quality of fluorotic enamel matrix formed during exposure was not different from that of normal enamel matrix. Based on the observation that this fluorotic matrix was able to mineralize after removal of fluoride from the culture medium, Lyaruu *et al.* (1989a, 1987c) suggested that there was no avid binding of fluoride to the enamel matrix and that this fluorotic matrix was still capable of forming enamel crystals. These results are contrary to those of Hammarström (1971), Weatherell *et al.* (1975), and DenBesten *et al.* (1992), but in agreement with equilibrium experiments by Crenshaw *et al.* (1978).

To interpret the fluoride effects on enamel formation *in vivo*, a more accurate determination of actual plasma F^- levels after F^- intake under various conditions is needed. Many factors, such as F^- dose, method of administration, time of exposure, composition of the diet, bone metabolism, and acid-base status have been reported to influence plasma F^- levels and accordingly its effect on dental mineralization (Bronckers, 1986). The fact that plasma fluoride levels do not necessarily reflect the actual concentration of F^- at the site of action during amelogenesis also complicates matters. To avoid these problems, effects of fluoride on tooth development were directly studied on enamel formation in organ culture conditions (Bronckers *et al.*, 1984a,b; Bronckers and Wöltgens, 1985).

In Table 1 biochemical and histological effects obtained so far *in vitro* by minimal F^- concentration at early stages of amelogenesis during short-term (1-2 days) and long-term (4-8 days) treatment are summarized.

During long-term exposure to F^- the secretion of extracellular amelogenins was already inhibited at levels of 1-2 μMol F^- and higher and uptake of calcium at 10 μMol and higher. Both concentrations are in the same range as those found in plasma at which fluorosis can be observed during chronic ingestion (Ekstrand, 1987; Wöltgens *et al.*, 1989b).

On the other hand, short-term exposure of the secretory ameloblast to F^- enhanced enamel mineralization (uptake of ^{45}Ca and ^{32}P) at levels of 52 μMol (1 ppm) F^-. In addition, new matrix formed in organ culture during F^- treatment (designated as fluorotic matrix) fails to mineralize but the pre-existing crystallites of previously formed enamel (called pre-exposure enamel) become hypermineralized (Lyaruu *et al.*, 1989a). In this respect, it is interesting to mention that F^- entry into the enamel compartment is not under cellular control of the overlaying secretory ameloblasts (Bawden and Deaton, 1981) and that F^- uptake is independent of the presence of calcium in the extracellular environment (Bawden and Crenshaw, 1984). Thus, F^- can be assumed to have free access toward the enamel mineralization front, resulting in an increased incorporation of ionic calcium from the enamel fluid into the existing secretory stage enamel crystals, which leads to the hypermineralization of pre-exposure enamel. Not all secretory stage enamel is hypermineralized by F^- to the same degree. An inverse relationship can be observed between early secretory, secretory and late secretory stages regarding the amount of non-mineralized fluorotic enamel matrix, and the degree of fluoride-induced hypermineralization (Lyaruu *et al.*, 1989a). To explain these differences we speculate that variation in non-mineralized enamel matrix thickness produced during fluoride treatment will lead to variation in local fluoride concentration near the forming enamel crystals and determines the degree of hypermineralization there. Consequently, the F^--induced hypermineralization is most distinct at the initial and final stages of enamel deposition, when the rate of enamel matrix secretion is low.

The ultrastructural and microprobe results obtained so far (Lyaruu *et al.*, 1987c, 1989a,b) indicate that fluoride induces hypermineralization of preculture enamel by preferential enhancement of crystal growth in thickness at the expense of growth in length. An important recent paper by Miake *et al.* (1993) studied compositional and structural changes in the enamel mineral during secretory amelogenesis. Their results support the idea that initial mineralization comprises two events: the initial precipitation of thin ribbons (increase in crystal length) and the subsequent epitaxial growth of apatite crystals on the octacalcium phosphate-like precursor (increase in crystal thickness). We hypothesize that both these processes regulate the cleavage of the originally secreted surrounding enamel matrix. Aoba and Moreno (1991) demonstrated that the lower molecular weight amelogenins adsorb less to hydroxy-apatite crystals and are less soluble in enamel fluid than the high molecular weight amelogenins. The proportional increase of lower molecular amelogenins over time in their model explains the preference of enamel crystals for growing width-wise instead of length-wise at older secretory stages as shown in cultured tooth explants (Lyaruu *et al.*, 1989a) as well as in isolated pieces of developing enamel from pigs (Miake, *et al.*, 1993).

According to Brown *et al.* (1987), F^- stimulates transformation of octacalcium phosphate to hydroxy-apatite. Thus, assuming that octacalcium phosphate is the precursor mineral in enamel, F^- may favor crystal growth in width at the expense of crystal growth in length concomitantly with an increased cleavage of the original enamel matrix. This phenomenon is very pronounced near the youngest secretory ameloblasts and may lower calcium concentration in the enamel fluid between the cell and hypermineralizing enamel front to such extent that secretion of enamel matrix is impaired (cf. Wöltgens *et al.*, 1987). Under F^- exposure and in cooperation with young odontoblasts newly differentiated ameloblasts only continue to form mantle dentin – which in our view consists of non-collagenous matrix proteins of both ameloblast and odontoblast origin – that subsequently mineralizes. This mantle dentin is covered by some rudimentary enamel that does not yet increase in thickness because of lack of further enamel matrix secretion (Lyaruu *et al.*, 1989a). In this way exposure of tooth cultures to fluoride will create a distance between the forming dentin front and enamel front (Bronckers *et al.*, 1989). Due to direct or indirect cytotoxic effect of fluoride (e.g. caused by lowering of ionic calcium in the enamel fluid) secretory ameloblasts lose their Tomes' processes. Loss of Tomes' processes or preventing their formation at the initial stage also prevents formation of rod/interrod structures of the forming enamel resulting in a layer of aprismatic enamel.

Young ameloblasts can recover from exposure to F^-: they resume matrix secretion and mineralization of the *in vitro* formed non-mineralized fluorotic enamel matrix when fluoride is removed from the culture medium (Lyaruu *et al.*, 1987c). This recovery process is enhanced when extra calcium is added to the medium (Table 2). Only the hypermineralized enamel remains irreversible. Generally the orientation of the new enamel crystals within the matrix depends on the degree of hypermineralization of the fluorotic enamel (Lyaruu *et al.*, 1987c; Bronckers *et al.*, 1989). An example of these fluoride effects is illustrated in a high power electron micrograph of the cervical loop region of a developing hamster tooth germ (Fig. 4). It shows highly hypermineralized aprismatic enamel induced by the presence of fluoride in the culture medium during the initial 24 h of culture (Fig. 4A). This layer is deposited *in vivo* before the onset of fluoride treatment *in vitro* (pre-exposure enamel, Lyaruu *et al.*, 1989a). During exposure fluorotic enamel matrix is formed that fails to mineralize but resumes mineralization when fluoride is removed from the culture medium or medium calcium is enhanced (Bronckers *et al.*, 1989). The orientation of the new enamel crystals is more or less parallel to the surface of the underlying fluorotic enamel.

Taken together we hypothesize that F^- acts directly on the existing enamel crystals thereby promoting the conversion of octacalcium phosphate into hydroxy-apatite and enhancing calcium incorporation into the crystal lattice. This process consumes calcium ions from the enamel fluid and causes a local hypocalcemia between the mineralization front and Tomes' process of the secretory ameloblast. It is this hypocalcemia in the enamel fluid below the actual level of 10^{-2} mMol (Aoba and Moreno, 1987; Bawden, 1989) that subsequently impairs mineralization of the fluorotic matrix. A strong argument in favor of this concept is our observation that if along with F^- the medium calcium levels are enhanced, these layers of fluorotic matrix do mineralize, suggesting that local hypocalcemia is indeed responsible for the failure of fluorotic matrix to mineralize (Bronckers *et al.*, 1989).

Transitional ameloblasts also appear to be very sensitive to short-term exposure to F^- (levels 4-8 μMol and higher) but F^- has no direct histological effects on resorbing ameloblasts during maturation *in vitro* (Wöltgens and Lyaruu, 1988). In agreement with the findings of Lange *et al.* (1986) and Simmelink and Lange (1989) for rat enamel and more recently of Kierdorf *et al.* (1993) for deer, Lyaruu *et al.* (1989b) recorded that a high fluoride dose injected in hamster always resulted in cyst formation under a layer of hypermineralized pre-exposed enamel. These cysts were however produced not by all but by certain isolated transitional ameloblasts, that became degenerative whilst other cells of the same developmental stage remained unaffected. These cysts may have been caused by the sudden increase in hydrostatic pressure in the enamel fluid due to enhanced generation of water molecules during the F^--induced transformation of octacalcium phosphate into hydroxy-apatite.

In conclusion, the use of an organ culture system has proved to be a valuable technique for acquiring and quantifying direct effects of modulators of enamel mineralization in developing tooth explants. Alkaline phosphatase has been implicated in dental mineralization for many years, although functional studies have not been reported. Experiments with l-pBTM (a specific inhibitor of alkaline phosphatase) in organ culture suggest that alkaline phosphatase stimulates dental mineralization indirectly through the modulation of phosphate supply and/or by eliminating phosphate esters known to inhibit mineralization. In stages before mineralization alkaline phosphatase may also be involved in phosphorylation of organic macromolecules. Organ culture experiments also reveal that for dental mineralization, the level of calcium in the medium is more crucial than that of phosphate, particularly for enamel formation. When medium calcium levels are low (about 1 mM) pre-ameloblasts fail to become secretory, in contrast to pre-odontoblasts and no *de novo* mineralization will occur, not even in the secreted predentin.

F^- induces many adverse effects on enamel development, but the mechanisms are poorly understood. Organ culture experiments suggest that many of the fluoride effects on amelogenesis can be explained by a state of hypocalcemia in the enamel fluid present between the ameloblast and the mineralization front caused by F^--stimulated hydroxy-apatite formation on the crystal lattices of the pre-existing enamel crystals. Most fluoride-induced effects can be reversed selectively by adding calcium but not phosphate to the medium. Only the hypermineralization of enamel crystals induced by fluoride treatment remains irreversible.

References

ALI, S.I. (1976). Analysis of matrix vesicles isolated from epiphysial cartilage. *Fed. Proc. 35:* 135-142.

AMELOOT, P.C., COOMANS, D., SMEYERS-VERBEKE, J. and BOUTE, P. (1987). Effect of different combinations of calcium, magnesium and phosphate on the inorganic composition of rat molars *in vitro*. *Arch. Oral Biol. 32:* 631-636.

AOBA, T. and MORENO, E.C. (1987). The enamel fluid in early secretory stage of porcine amelogenesis: chemical composition and saturation with respect to enamel mineral. *Calcif. Tissue Int. 41:* 86-94.

AOBA, T. and MORENO, E.C. (1991). Structural relationship of amelogenin proteins to their regulatory function of enamel mineralization. In *Surface Reactive Peptides and Polymers* (Eds. C.S. Sikes and A.P. Wheeler). American Chemical Society Symposium Series 444, New York, pp. 86-107.

BAWDEN, J.W. (1989). Calcium transport during mineralization. *Anat. Rec. 224:* 226-233.

BAWDEN, J.W. and CRENSHAW, M.A. (1984). Effect of net calcium uptake on net fluoride uptake in developing rat molars. *J. Dent. Res. 63:* 642-645.

BAWDEN, J.W. and DEATON, T.G. (1981). *In vitro* study of the cellular control of ^{18}F uptake in the enamel of developing rat teeth. *Arch. Oral Biol. 26:* 487-490.

BAWDEN, J.W. and WENNBERG, A. (1977). *In vitro* study of cellular influence on ^{45}Ca uptake in developing rat enamel. *J. Dent. Res. 56:* 313-319.

BAWDEN, J.W., CRENSHAW, M.A., TAKANO, Y. and HAMMARSTRÖM, L. (1982). Ion transport through the enamel organ. An update. *J. Dent. Res. 61:* 1552-1554.

BAWDEN, J.W., DEATON, T.G. and CRAWFORD, B.P. (1992). Fluoride and calcium content of enamel organ, muscle, liver and plasma in rats. *Caries Res. 26:* 263-267.

BAWDEN, J.W., ROZELL, B., WURTZ, D., FOUDA, N. and HAMMARSTRÖM, L. (1994). Distribution of protein kinase Cα and accumulation of extracellular Ca^{2+} during early dentin and enamel formation. *J. Dent. Res. 73:* 1429-1436.

BERDAL, A., HOTTON, D., PIKE, W., MATHIEU, H. and DUPRET, J.M. (1993). Cell- and stage- specific expression of vitamin D receptor and calbindin genes in rat incisor: regulation by 1,25- dihydroxyvitamin D_3. *Dev. Biol. 155:* 172-179.

BERNARD, G.W. (1972). Ultrastructural observations of initial calcification in dentin and enamel. *J. Ultrastruct. Res. 41:* 1-17.

BISAZ S., RUSSEL, R.G.G. and FLEISCH, H. (1968). Isolation of inorganic pyrophosphate from bovine and human teeth. *Arch. Oral Biol. 13:* 683-696.

BONTING, S.J. and NUKI, K. (1963). Comparative studies of the developing hamster tooth by quantitative ultramicrochemical technique and by histochemical staining methods. *Ann. Histochim. 8:* 79-88.

BORKE, J.L., ZAKI, A.E., EISENMANN, D.R., ASHRAFI, S.H., ASHRAFI, S.S. and PENNISTON, J.T. (1993). Expression of plasma membrane Ca^{++} pump epitopes parallels the progression of enamel and dentin mineralization in rat incisor. *J. Histochem. Cytochem. 41:* 175-181.

BRONCKERS, A.L.J.J. (1981). *The Development of Hamster Molars in Vivo and in Vitro.* PhD Thesis, Vrije Universiteit, Amsterdam.

BRONCKERS, A.L.J.J. (1986). Fluoride action on biological processes during enamel development. In *Tooth Development and Caries,* Vol. I (Eds. F.C.M. Driessens and J.H.M. Wöltgens). CRC Press, Inc., Boca Raton, pp. 53-83.

BRONCKERS, A.L.J.J. and WÖLTGENS, J.H.M. (1985). Short-term effects of fluoride on biosynthesis of enamel-matrix proteins and dentine collagens and on mineralization during hamster tooth-germ development in organ culture. *Arch. Oral Biol. 2:* 181-191.

BRONCKERS, A.L.J.J., BERVOETS T.J.M., LYARUU D.M. and WÖLTGENS J.H.M. (1989). High levels of calcium but not inorganic phosphate antagonize fluoride toxicity on secretory amelogenesis in hamster tooth germs *in vitro*. *Arch. Oral Biol. 34:* 625-636.

BRONCKERS, A.L.J.J., JANSEN, L.L. and WÖLTGENS, J.H.M. (1984a). A histological study of the short-term effects of fluoride on enamel and dentine formation in hamster tooth-germs in organ culture *in vitro*. *Arch. Oral Biol. 29:* 803-810.

BRONCKERS, A.L.J.J., JANSEN, L.L. and WÖLTGENS, J.H.M. (1984b). Long term (8 days) effects of exposure to low concentrations of fluoride on enamel formation in hamster tooth-germs in organ culture *in vitro*. *Arch. Oral Biol. 29:* 811-819.

BRONCKERS, A.L.J.J., LYARUU D.M., BERVOETS T.J.M. and WÖLTGENS J.H.M. (1988). Autoradiographic, ultrastructural and biosynthetic study of the effect of colchicine on enamel matrix secretion and enamel mineralization in hamster tooth germs *in vitro*. *Arch. Oral Biol. 33:* 7-16.

BROWN, W.E., EIDELMAN, N. and TOMAZIC, B. (1987). Octacalcium phosphate as a precursor of biomineral formation. *Adv. Dent. Res. 1:* 306-314.

BUTLER, W.T. (1992). Structure and function of mineralized tissue components. In *Chemistry and Biology of Mineralized Tissues* (Eds. H. Slavkin and P. Price). Excerpta Medica, Amsterdam, pp. 165-169.

CRENSHAW, M., WENNBERG, A. and BAWDEN, J. (1978). Fluoride-binding by the organic matrix of developing bovine enamel. *Arch. Oral Biol. 23:* 285-287.

DENBESTEN, P.K., HEFFERNAN, L.M., FEATHERSTONE, J.D.B. and SHIELDS, C.P. (1992). Fluoride binding by matrix proteins in rat mineralizing tissue. *Arch. Oral Biol. 37:* 459-462.

DEPORTER, D.A. (1977). The early mineralization of enamel. Fine structural localization of calcium with K-pyroantimonate technique. *Calcif. Tissue Int. 34:* 271-274.

DEPORTER, D.P. and TENCATE, A.R. (1976). Fine structural localisation of alkaline phosphatase in relation to enamel formation. *Arch. Oral Biol. 21:* 7-9.

DEUTSCH, D. and GEDALIA, I. (1982). Fluoride concentration in human enamel. *Caries Res. 16:* 428-432.

DOGTEROM, A.A., LYARUU, D.M., DODERER, A. and WÖLTGENS, J.H.M. (1984). Partial purification and some characteristics of hamster molar alkaline phosphatase. *Experientia 40:* 1259-1262.

DULCE, H.J. (1960). Zur Biochemie der Verknöcherung. II. Enzymaktivität im hyalinen Knorpel, im verknöcherenden Knorpel und im Knochen. *Hoppe-Seylers Z. Physiol. Chem. 319:* 272-278.

EATON, R.H. and MOSS, D.W. (1968). Partial purification and some properties of human bone alkaline phosphatase. *Enzymologia 35:* 31-39.

EKSTRAND, J. (1987). Pharmacokinetic aspects of topical fluorides. *J. Dent. Res. 66:* 1061-1065.

ENGEL M.B. (1981). Microprobe analysis of calcifying matrices and formative cells in developing mouse molars. *Histochemistry 72:* 443-452.

ERICSSON S.Y. (1977). Cariostatic mechanisms of fluorides: clinical observations. *Caries Res. 11 (Suppl. 1):* 2-41.

FELIX, R. and FLEISCH, H. (1976). Pyrophosphatase and ATP-ase of isolated cartilage matrix vesicles. *Calcif. Tissue Res. 22:* 1-7.

FERGUSON, H.W. and HARTLES, R.L. (1966). The effects of diets deficient in calcium or phosphorus in the presence or absence of supplements of vitamin D on the incisor teeth and bone of adult rats. *Arch. Oral Biol. 11:* 1345-1364.

FLEISCH, H. (1964). Role of nucleation and inhibition in calcification. *Clin. Orthop. 32:* 170-180.

GLICK, P.L. (1979). Pattern of enamel maturation. *J. Dent. Res. 58:* 883-892.

HAMMARSTRÖM, L. (1971). Distribution in developing rat enamel of simultaneously injected fluoride and calcium. *Scand. J. Dent. Res. 79:* 369-376.

HUI, M., HU, M. and TENENBAUM, H.C. (1993). Changes in cell adhesion and cell proliferation are associated with expression of tissue non-specific alkaline phosphatase. *Cell Tissue Res. 274:* 429-437.

KIERDORF, U., KIERDORF, H. and FEJERSKOV, O. (1993). Fluoride-induced developmental changes in enamel and dentine of European roe deer (*Capreolus Capreolus* L.) as a result of environmental pollution. *Arch. Oral Biol. 38:* 1071-1081.

KLEIN, B.Y., GAL, I. and SEGAL, D. (1993). Studies of the levamisole inhibitory effect on rat stromal-cell commitment to mineralization. *J. Cell. Biochem. 53:* 114-124.

KOGAYA, Y. and FURUHASHI, K. (1986). The differences in calcium distribution pattern between preodontoblasts and preameloblasts in developing rat molar tooth germs. *Calcif. Tissue Int. 39:* 78-85.

KRUGER, B.J. (1968). Ultrastructural changes in ameloblasts from fluoride treated rats. *Arch. Oral Biol. 13:* 969-977.

KRUGER, B.J. (1970). The effects of different levels of fluoride on the ultrastructure of ameloblasts in rats. *Arch. Oral Biol.15:* 109-114.

LANGE, A., EKSTRAND, J. and HAMMARSTRÖM, L. (1986). Fluoride-induced cystic changes in the enamel organ of the rat molar. *J. Oral Pathol. 15:* 87-92.

LARMAS, M. and THESLEFF, I. (1980). Biochemical study of changes in non-specific alkaline phosphomonoesterase activity during mouse tooth ontogeny. *Arch. Oral Biol. 25:* 791-797.

LEBLOND, C.P. and WARSHAWSKY, H. (1979). Dynamics of enamel formation in the rat incisor. *J. Dent. Res. 58B:* 950-975.

LOZUPONE, E. and FAVIA, A. (1994). Morphometric analysis of the deposition and mineralization of enamel and dentine from rat incisor during the recovery phase following a low-calcium regimen. *Arch. Oral Biol. 39:* 409-416.

LYARUU, D.M. (1983). *The Role of Alkaline Phosphatase and Ionic Calcium in HamsterTooth Germs in Vivo and in Vitro with Special Reference to Early Amelogenesis*. PhD Thesis, Vrije Universiteit, Amsterdam.

LYARUU, D.M., BLIJLEVEN, N., HOEBEN SCHORNAGEL, K., BRONCKERS, A.L.J.J. and WÖLTGENS, J.H.M. (1989a). X-ray Micro-analysis of the mineralization patterns in developing enamel in hamster tooth germs exposed to fluoride *in vitro*. *Adv. Dent. Res. 3:* 212-220.

LYARUU, D.M., BRONCKERS, A.L.J.J., WÖLTGENS, J.H.M. and HOEBEN SCHORNAGEL, K. (1987a). Localization of cellular calcium in differentiating ameloblasts and its relationship to the early mineralization process in mantle dentine and enamel, in hamster tooth germs *in vitro*. *Adv. Dent. Res. 1:* 202-213.

LYARUU, D.M., LENGLET, W.J.M., WÖLTGENS, J.H.M. and BRONCKERS, A.L.J.J. (1989b). Micro PIGE determination of fluorine distribution in developing hamster tooth germs. *J. Histochem. Cytochem. 37:* 581-587.

LYARUU, D.M., VAN DUIN, M.A., BERVOETS, T.J.M., WÖLTGENS, J.H.M. and BRONCKERS, A.L.J.J. (1995). Effects of vincristine on the developing hamster tooth germ *in vitro*. *Connect. Tissue Res. 31:* 1-9.

LYARUU, D.M., WÖLTGENS, J.H.M. and BERVOETS, T.J.M. (1987b). Effect of alkaline phosphatase inhibitors by l-p-bromotetramisole on the formation of TCA-insoluble ^{32}Phosphate in non-mineralizing and mineralizing hamster tooth germs *in vitro*. *Arch. Oral Biol. 32:* 429-433.

LYARUU, D.M., WÖLTGENS, J.H.M., BERVOETS, T.J.M. and DOGTEROM, A.A. (1983). Studies of alkaline phosphatase inhibition by p-bromotetramisole in non-mineralizing and mineralizing neonatal hamster tooth germs *in vitro*. *J. Biol. Buccale 11:* 347-353.

LYARUU, D.M., WÖLTGENS. J.H.M., BRONCKERS, A.L.J.J. and DE JONG, M. (1987c).Ultrastructural study of the *in vitro* recovery of enamel matrix mineralization in hamster tooth germs pre-exposed to fluoride in organ culture during the secretory phase of amelogenesis. *Arch. Oral Biol. 32:* 107-115.

MIAKE, Y., SHIMODA, S., FUKAE, M. and AOBA, T. (1993). Epitaxial overgrowth of apatite crystals on thin-ribbon precursor at early stages of porcine enamel mineralization. *Calcif. Tissue Int. 53:* 249-256.

NEUMAN, W.F. and NEUMAN W.M. (1958). *The Chemical Dynamics of Bone Mineral*. Chicago University Press, Chicago.

NIKIFORUK, G. and FRASER, D. (1979). Etiology of enamel hypoplasia and interglobular dentin: the roles of hypocalcemia and hypophosphatemia. *Metab. Bone Dis. Relat. Res. 2:* 17-23.

OIDA, S., GOSEKI, M. and SASAKI, S. (1985). Purification and subunit structure of alkaline phosphatase from bovine enamel organ. *Arch. Oral Biol. 30:* 193-196.

PINDBORG, J.J. (1982). Aetiology of developmental enamel defects not related to fluorosis. *Int. Dent. J. 32:* 123-134.

RAHKAMO A. (1974). Mineralization of rat teeth in tissue culture. *Proc. Finn. Dent. Soc. 70:* 165-170.

REITH, E.J. (1983). A model for transcellular transport of calcium based on membrane fluidity and movement of calcium carriers within the more fluid microdomains of the plasma membrane. *Calcif. Tissue Int. 35:* 129-134.

REITH, E.J. and BOYDE, A. (1985).The pyroantimonate reaction and transcellular transport of calcium in rat molar enamel organs. *Histochemistry 83:* 539-543.

ROBISON R., MacLEOD, M. and ROSENHEIM, A.H. (1930). The possible significance of hexose phosphoric esters in ossifications. IX. Calcification *in vitro*. *Biochem. J. 24:* 1927-1941.

SCHROEDER, H.E. (1991). *Oral Structural Biology*. Thieme Medical Publisher, New York.

SIMMELINK, J.W. and LANGE, A. (1986). Ultrastructure of altered rat enamel beneath fluoride-induced cysts. *J. Oral Pathol. 15:* 155-161.

SLAVKIN, H.C. (1975). The isolation and characterization of calcifying and non-calcifying matrix vesicles from dentine. *Coll. Inter. du CNRS no. 230, Physico-chimie et cristall. des apatites d'intérêt. biol.* pp. 161-177.

SPEIRS, R.L. (1978). Fluoride concentration in tooth germs of permanent teeth of the domestic pig. *Arch. Oral Biol. 23:* 1019-1021.

STRATMANN, U., BARCKHAUS, R.H., LYARUU, D.M., WÖLTGENS, J.H.M., WESSELING, G. and BAUMEISTER, A. (1991). Electroprobe X-ray micro analysis of calcium and phosphorus distribution in developing hamster tooth germs *in vitro* and *in vivo*. *Acta Anat. 140:* 343-349.

TAKANO, Y. (1992). Histochemical demonstration and microanalysis of possible calcium binding sites in the enamel organ of rat incisors. *Scan. Microsc. 6:* 773-784.

TAKANO, Y., OZAWA, H. and CRENSHAW, M.A. (1986). Ca-ATPase and ALP-ase activities at the initial calcification site of dentin and enamel in the rat incisor. *Cell Tissue Res. 243:* 91-99.

TOGARI, A., ARAKAWA, S., ARAI, M. and MATSUMOTO, S. (1993). Alteration of *in vitro* bone metabolism and tooth formation by Zinc. *Gen. Pharmacol. 24:* 1133-1140.

TOJYO, Y. (1983). A comparison of the alkaline phosphatase of rat dental pulp, bone, kidney, liver and intestine. *Arch. Oral Biol. 28:* 103-107.

TOJYO, Y., SAIDA, K. and SUZUKI, A. (1981). Polyacrylamide electrophoresis of the alkaline phosphatase of rat dental pulp. *Arch. Oral Biol. 26:* 1099-1100.

TROS, G.H.J., LYARUU, D.M. and VIS, R.D. (1993). Quantitative analysis of fluoride-induced hypermineralization of developing enamel in neonatal hamster tooth germs. *Nucl. Instrum. Meth. B 83:* 255-265.

VAN BELLE, H. (1972). Kinetics and inhibition of alkaline phosphatases from canine tissues. *Biochim. Biophys. Acta 289:* 158-165.

WALTON, R.E. and EISENMANN, D.R. (1974). Ultrastructural examination of various stages of amelogenesis in the rat following parenteral fluoride administration. *Arch. Oral Biol. 19:* 171-182.

WEATHERELL, J.A., DEUTSCH, D., ROBINSON C. and HALLSWORTH, A.S. (1975). Fluoride concentration in developing enamel. *Nature 256:* 230-232.

WIGGLESWORTH, D.J. and HAYWARD, A.F. (1973). The ultrastructure of dentinogenesis and amelogenesis in rat molar tooth germs grown as organ cultures *in vitro*. *Z. Zellforsch. 138:* 171-186.

WÖLTGENS, J.H.M. (1971). *Role of Inorganic Pyrophosphatase in Dental Mineralization.* PhD Thesis, University of Nijmegen, Nijmegen.

WÖLTGENS, J.H.M. (1974). Inorganic pyrophosphatase in mineralizing hamster molars IV. Modification of the inhibition of EDTA, diphosphonates and l-ascorbic acid by Zn^{2+} and Cd^{2+}. *Calcif. Tissue Res. 16:* 201-207.

WÖLTGENS, J.H.M. and BERVOETS, T.J.M. (1978). Electrophoretic distribution of calcium, protein, alkaline phosphatase and inorganic pyrophosphatase from ectodermal and mesodermal tissue of developing bovine incisor. *Arch. Oral Biol. 23:* 1027-1028.

WÖLTGENS, J.H.M. and LYARUU, D.M. (1988). The effects of fluoride on various stages of enamel formation *in vitro*. *J. Fluorine Chem. 41:* 71-78.

WÖLTGENS, J.H.M., BERVOETS, T.J.M., BRONCKERS, A.L.J.J. and LYARUU, D.M. (1982). Organ culture of tooth germs: relationship between alkaline phosphatase and mineralization *in vitro*. *J. Biol. Buccale 10:* 191-198.

WÖLTGENS, J.H.M., BERVOETS, T.J.M., LYARUU, D.M. and BRONCKERS, A.L.J.J. (1989a). The effects of cadmium on the p-NPP-ase and PP_i-ase activities of alkaline phosphatase in developing hamster tooth germs. *Arch. Oral Biol. 84:* 591-592.

WÖLTGENS, J.H.M., BONTING, S.L. and BIJVOET, O.L.M. (1970). Relationship of inorganic pyrophosphatase and alkaline phosphatase activities in hamster molars. *Calcif. Tissue Res. 5:* 333-343.

WÖLTGENS, J.H.M., BONTING, S.L. and BIJVOET, O.L.M. (1971). Relationship between alkaline phosphatase, inorganic pyrophosphatase and l-ascorbic acid in calcifying hamster molars. *Clin. Orthop. 78:* 296-302.

WÖLTGENS, J.H.M., ETTY, E.J., NIEUWLAND, W.M.D. and LYARUU, D.M. (1989b). Use of fluoride by young children and prevalence of mottled enamel. *Adv. Dent. Res. 3:* 177-182.

WÖLTGENS, J.H.M., LYARUU D.M., BRONCKERS, A.L.J.J. and BERVOETS, T.J.M. (1987). Effects of calcium and phosphate on secretion of enamel matrix and its subsequent mineralization. *Adv. Dent. Res. 1:* 196-202.

WÖLTGENS, J.H.M., LYARUU, D.M. and BERVOETS, T.J.M. (1991). Possible functions of alkaline phosphatase in dental mineralization: cadmium effects. *J. Biol. Buccale 19:* 125-128.

WÖLTGENS, J.H.M., LYARUU, D.M. and BRONCKERS, A.L.J.J. (1985). Effect of l-p-bromotetramisole on phosphatase in neonatal hamster bone and tooth germs at different pH. *J. Biol. Buccale 13:* 3-10.