Dental Fluorosis: Chemistry and Biology

T. Aoba and O. Fejerskov

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DENTAL FLUOROSIS: CHEMISTRY AND BIOLOGY

T. Aoba[1]*
O. Fejerskov[2]

[1]The Nippon Dental University, Department of Pathology, 1-9-20 Fujimi, Chiyoda-ku, Tokyo 102, Japan; [2]The Royal Dental College, Faculty of Health Sciences, Aarhus University, Vennelyst Boulevard, 8000 Aarhus C, Denmark; *corresponding author, pathology-ndu@tokyo.ndu.ac.jp

ABSTRACT: This review aims at discussing the pathogenesis of enamel fluorosis in relation to a putative linkage among ameloblastic activities, secreted enamel matrix proteins and multiple proteases, growing enamel crystals, and fluid composition, including calcium and fluoride ions. Fluoride is the most important caries-preventive agent in dentistry. In the last two decades, increasing fluoride exposure in various forms and vehicles is most likely the explanation for an increase in the prevalence of mild-to-moderate forms of dental fluorosis in many communities, not the least in those in which controlled water fluoridation has been established. The effects of fluoride on enamel formation causing dental fluorosis in man are cumulative, rather than requiring a specific threshold dose, depending on the total fluoride intake from all sources and the duration of fluoride exposure. Enamel mineralization is highly sensitive to free fluoride ions, which uniquely promote the hydrolysis of acidic precursors such as octacalcium phosphate and precipitation of fluoridated apatite crystals. Once fluoride is incorporated into enamel crystals, the ion likely affects the subsequent mineralization process by reducing the solubility of the mineral and thereby modulating the ionic composition in the fluid surrounding the mineral. In the light of evidence obtained in human and animal studies, it is now most likely that enamel hypomineralization in fluorotic teeth is due predominantly to the aberrant effects of excess fluoride on the rates at which matrix proteins break down and/or the rates at which the by-products from this degradation are withdrawn from the maturing enamel. Any interference with enamel matrix removal could yield retarding effects on the accompanying crystal growth through the maturation stages, resulting in different magnitudes of enamel porosity at the time of tooth eruption. Currently, there is no direct proof that fluoride at micromolar levels affects proliferation and differentiation of enamel organ cells. Fluoride does not seem to affect the production and secretion of enamel matrix proteins and proteases within the dose range causing dental fluorosis in man. Most likely, the fluoride uptake interferes, indirectly, with the protease activities by decreasing free Ca^{2+} concentration in the mineralizing milieu. The Ca^{2+}-mediated regulation of protease activities is consistent with the *in situ* observations that (a) enzymatic cleavages of the amelogenins take place only at slow rates through the secretory phase with the limited calcium transport and that, (b) under normal amelogenesis, the amelogenin degradation appears to be accelerated during the transitional and early maturation stages with the increased calcium transport. Since the predominant cariostatic effect of fluoride is not due to its uptake by the enamel during tooth development, it is possible to obtain extensive caries reduction without a concomitant risk of dental fluorosis. Further efforts and research are needed to settle the currently uncertain issues, *e.g.*, the incidence, prevalence, and causes of dental or skeletal fluorosis in relation to all sources of fluoride and the appropriate dose levels and timing of fluoride exposure for prevention and control of dental fluorosis and caries.

Key words. Fluorosis, fluoride, enamel, matrix proteins, proteases, mineralization, caries prevention.

(1) Introduction

Fluoride plays a key role in the prevention and control of dental caries. Ever since the excellent work of Dean and his collaborators (Dean 1934, 1942; Dean and Elvovo, 1935), in which the association between fluoride in drinking water and the occurrence of disturbances of tooth formation (mottling of enamel or dental fluorosis), as well as a concomitant reduction in caries experience, was demonstrated, dental fluorosis has been a central issue in all programs seeking to harness the unique property of fluorides to control and prevent caries. In the middle of the previous century, the paradigm was that, to exert its maximum cariostatic effect, fluoride had to become incorporated into dental enamel during development, and hence it was inevitable to have a certain prevalence and severity of fluorosis in a population to minimize the prevalence and severity of caries among children. Dental fluorosis was then regarded as an unfortunate side-effect of fluoride's caries-protective benefits, and attempts to "play down" the possible toxic effect of fluoride on developing dental enamel often led the dental profession to present dental fluorosis as merely a cosmetic problem.

Much of what is perpetuated world-wide in caries-preventive programs today is derived from the beliefs and paradigms concerning fluorides generated in the 1950s and '60s, but now we are at a time in science where "evidence-based medicine" is becoming a central issue for evaluating the scientific achievements during the last decades concerning our understanding of how fluoride affects mineralizing and mineralized dental hard tissues. The last 25 years have presented major breakthroughs in basic research on how fluoride affects the mineralization of teeth and how fluoride exerts its cariostatic effect (for reviews, see Fejerskov *et al*., 1981; Ten Cate and Featherstone, 1996). It is remarkable, however, that the dramatic decline in dental caries which we have witnessed in many different parts of the world (for reviews, see Glass, 1982; Fejerskov and Baelum, 1998) has occurred without the dental profession being fully able to explain the relative role of fluoride in this intriguing process. It is a common belief that the wide distribution of fluoride from toothpastes may be a major explanation (Bratthall *et al*., 1996), but serious attempts to assess the role of fluoridated toothpastes

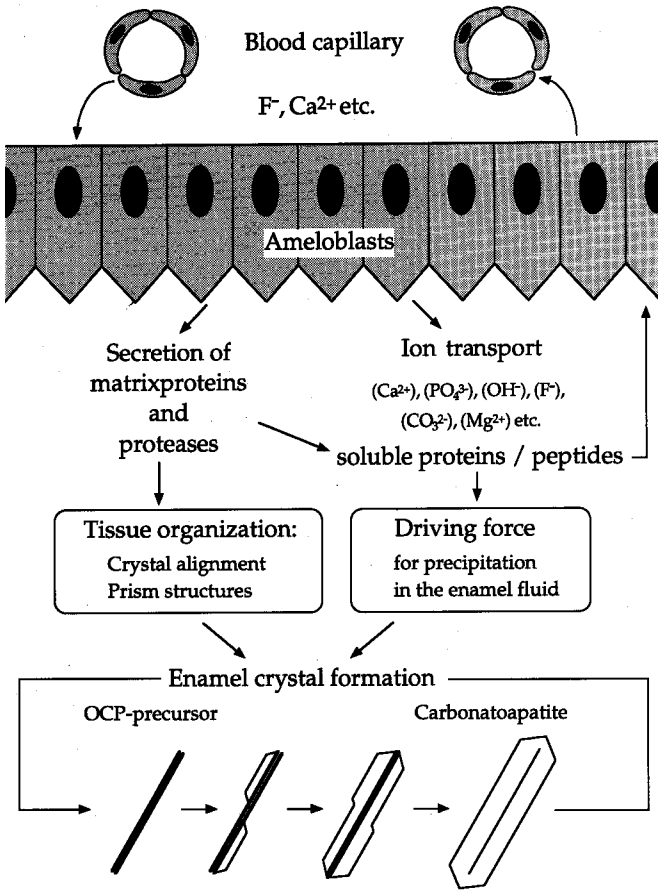

Figure 1. Schematic illustration of the events relevant to early enamel mineralization. The ameloblasts regulate mass transport (*e.g.*, ions and organic matter) from blood circulation to the extracellular space and *vice versa*. The mineralizing environment is comprised of the secreted matrix proteins and proteases, and varieties of ions and soluble moieties. Post-secretory processing of the matrix proteins yields soluble moieties prior to removal from the forming enamel. Tissue organization is guided by the molecular architecture of the matrix proteins, while the driving force for precipitation is determined by activities of common ions in the enamel fluid. The resulting crystal formation is characterized by the initial precipitation of acidic precursors, thin ribbons in morphology, and the consecutive epitaxial overgrowth of carbonato-apatite.

have been able to attribute, at best, about 40-50% of the caries reduction to these fluoride products (Marthaler, 1990; Scheie, 1992). This is not surprising, if one takes into account the fact that dental caries is not the result of fluoride deficiency. Because of its unique electrochemical behavior, fluoride is the most potent agent which influences de- and remineralizing processes within a certain pH interval. It is possible to achieve a degree of undersaturation with respect to biological carbonated apatites which then dissolve, while the presence of fluoride at the very interface between solid and liquid may result in a relative degree of supersaturation with respect to fluoridated apatite (Larsen, 1975). So the net outcome under these conditions would be the dissolution of carbonated apatite with concomitant precipitation of a fluoridated hydroxyapatite in the surface zone, generating a classic subsurface type of lesion. This also explains why fluoride is accumulated in the surface zone of caries lesions *in vivo* (Hallsworth and Weatherell, 1969).

By 1981, it was therefore possible to propose a paradigm shift concerning the cariostatic mechanisms of fluorides (Fejerskov *et al*., 1981), in which it was argued that the predominant, if not the entire, explanation for how fluoride controls caries lesion development lies in its topical effect on de- and remineralization processes taking place at the interface between the tooth surface and the oral fluids. This concept has gained wide acceptance (Ten Cate and Duijsters, 1983; Ten Cate and Featherstone, 1991).

During the same period, it also became evident that the way dental fluorosis had been perceived was not in accordance with the histopathological changes in fluorosed human teeth. First, it became apparent that dental fluorosis was the result of a gradual increase in hypomineralization of teeth (Fejerskov *et al*., 1974), and, based on ultrastructural studies of human dental fluorosis, it was remarkable how similar the subsurface hypomineralization was in certain stages in normal human enamel maturation (Fejerskov *et al*., 1975, 1977). Moreover, the pits occurring in more severe cases of human dental fluorosis had hitherto been considered as hypoplasias resulting from a direct effect of fluoride on secreting ameloblasts, but studies clearly indicated that the pits were post-eruptive and reflected damage to the severely porous and hypomineralized outer enamel (Thylstrup and Fejerskov, 1979). The half-a-century-long debate about difficulties in recording early stages of dental fluorosis in man were resolved in an excellent review by Myers (1983), and, based on a histological understanding of the features of dental fluorosis, Thylstrup and Fejerskov (1979) suggested a valid and sensitive clinical diagnostic system which expanded on the excellent work of Dean. Finally, in re-assessments of what was known in the literature concerning the relationship between fluoride dose and the development of dental fluorosis, it was possible to estimate more precisely the actual dose of fluoride needed to cause increasing severity of fluorosis in a population (Fejerskov *et al*., 1994). These estimates, which were later shown very precisely, were able to predict the risk of developing dental fluorosis from different fluoride tablet regimes used in the US and Scandinavia at the time (Fejerskov *et al*., 1996).

During the last 10-15 years, there has been a revolution in our understanding of the biological processes leading to tooth development and the mineralization of the dental hard tissues. Much of this development is not widely known and appreciated within the dental profession, and the aim of this review is to provide an up-to-date re-assessment of the available literature on the effect of fluoride on the biomineralization processes in the dental hard tissues. We will demonstrate in the review that a better understanding of the effects of fluoride on these processes in dental enamel can be gained through very basic studies on the molecular events occurring during enamel formation. At the end of this review, we will therefore try to suggest future avenues for important research projects which ultimately would lead to data necessary for the further development of efficient and cost-effective public health measures which can control dental caries. Despite the decline in caries incidence in child populations in recent years, it should be noted that dental caries is still the predominant cause of tooth loss in any population world-wide.

(2) Structural Matrix Proteins and Proteases as Essential Constituents of Developing Enamel

Enamel formation is a complex process involving cellular proliferation and differentiation through the sequential epithelial-mesenchymal interactions, secretion of the tissue-specific matrix pro-

teins, transport of the ions including calcium and fluoride, and precipitation and alignment of enamel crystals through the multiple interactions between organic and inorganic molecules (Fig. 1). These processes progress over long periods in the fluid microenvironment segregated from the circulating blood (Aoba and Moreno, 1987; Moreno and Aoba, 1987). In the last decade, great advances have been made regarding the identification and cloning of a family of structural matrix proteins and enamel resident proteases. The matrix proteins identified so far are amelogenins, ameloblastin (also called amelin/sheathlin), tuftelin, sulphated high-molecular-weight proteins, and enamelin (for reviews, see: Simmer and Fincham, 1995; Fincham *et al*., 1999). Amelogenins are the major ameloblast-specific gene products, comprising more than 90% of the proteins secreted by the ameloblasts. The importance of amelogenin proteins in enamel integrity has been verified in several families with different mutations in the X-chromosomal amelogenin gene (for review, see Wright, 1997). The current literature suggests that extracellular enamel proteins are degraded by a variety of proteases present within the enamel at specific times in development (for reviews, see: Zeichner-David *et al*., 1995; Bartlett and Simmer, 1999). The enamel resident proteases and their activities are generally identified in the soluble fraction isolated from the forming enamel (Shimizu and Fukae, 1983), so the enzyme-substrate reaction most likely takes place at solid (aggregated or crystal-associated proteins)-fluid interfaces or in the fluid containing soluble protein molecules (Fig. 2). In recent years, two groups of enamel proteases have been cloned: a matrix metalloproteinase named enamelysin, or MMP-20 (Bartlett *et al*., 1996, 1998), and a serine proteinase named enamel matrix serine proteinase-1, or EMSP1 (Simmer *et al*., 1998). Immunohistochemical studies have demonstrated that enamelysin is secreted by the secretory ameloblasts developing Tomes' process and that the physical location of enamelysin within the secretory enamel overlaps with the location of the secreted amelogenins and other matrix proteins (Fukae *et al*., 1998). In studies combining recombinant porcine amelogenin with recombinant porcine enamelysin (Ryu *et al*., 1999), it was proven that the recombinant enzyme produces a pattern of amelogenin cleavages virtually identical to those observed *in vivo*. This enzyme exhibits its optimal activity at neutral pH (around 7.2) and high calcium ion concentration (Fukae *et al*., 1998). In contrast to enamelysin, the EMSP1 message level and enzymatic activity are low or absent during the early stages of enamel development and are up-regulated during the later stages (Hu *et al*., 2000). These results suggest that EMSP1 plays a role in the degradation of enamel matrix proteins during the transition to maturation stages of amelogenesis. In addition, more exo- and endopeptidases and proteinase inhibitors are believed to participate in enamel maturation (Smid *et al*., 1990; Toyosawa *et al*., 1996), although their roles and functional significance remain to be elucidated. Currently, there is no unequivocal evidence that enamel proteases and their activities are critical to enamel formation or development of enamel fluorosis, because no proteinase genes have been linked to inherited diseases of enamel formation such as amelogenesis imperfecta. The construction of knockout or transgenic models is certainly a useful approach to delineate the roles of proteases in tooth morphogenesis, but no targeted knockouts of proteinase genes are yet known to result in enamel malformations resembling fluorotic enamel.

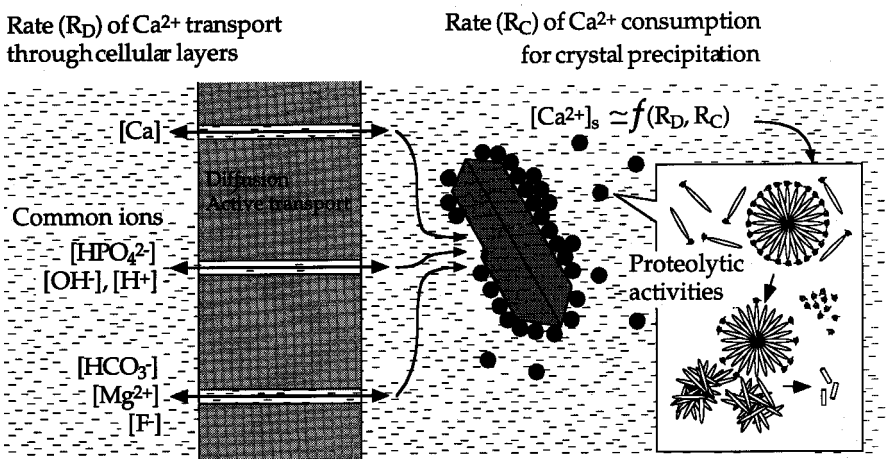

Figure 2. Schematic illustration of ion transport through the enamel organ layer and associated extracellular events taking place in the forming enamel. Ions transported through the cell layer are incorporated into the lattice positions. The precipitation rate is determined by the degree of supersaturation, fluid volume, crystal surface areas, protein coating, and the presence of inhibitors (*e.g.*, carbonate and Mg^{2+}) and promoter, *i.e.*, fluoride. As a result, the Ca^{2+} concentration in the fluid ($[Ca]_s$) surrounding the forming crystals is determined in principle by the balance between the rates of Ca^{2+} supply (R_D) and consumption for crystal precipitation (R_C). Proteolytic processing of the amelogenins and other proteins, possibly in a form of nanosphere aggregates, may depend on the $[Ca]_s$ levels.

(3) Post-secretory Enzymatic Processing of Enamel Matrix Proteins

The most distinctive feature of mammalian amelogenesis, in contrast to dentinogenesis and osteogenesis, is that the secreted matrix proteins are degraded *in situ* and removed almost completely from the tissue during the secretory through maturation stages. The enzymatic degradation of the amelogenins (Shimizu and Fukae, 1983), as well as other classes of enamel matrix proteins (Smith, 1998), occurs shortly after their secretion, so the full-length proteins are concentrated only in the narrow region of the secretory enamel adjacent to the ameloblasts. The resulting heterogenous population of proteolytic cleavage products remains relatively stable during the formation of full-thickness enamel (Aoba *et al*., 1987b; Brookes *et al*., 1995). After cessation of the secretion of the matrix proteins, their cleavage products are further degraded. This allows the enamel layer to attain its high degree of mineralization prior to tooth eruption. These temporally regulated processing of enamel proteins appears to be carried out by the separate classes of proteases, *e.g.*, enamelysin and EMSP1, with some overlap of function.

The post-secretory processings of amelogenins have been the most often investigated, so the scheme of the protein-protease interaction is becoming clear. The full-length amelogenins in mammals are known to have the conserved primary structures, particularly the hydrophobic and hydrophilic segments at the N- and C-termini, respectively (Aoba *et al*., 1992a,b; Brookes *et al*., 1994). The hydrophilic segment at the C-terminus has been shown to be exposed to the external liquid phase (Lau *et al*., 1987; Aoba *et al*., 1990a), and it was consistently shown that the initial cleavage of the amelogenin molecule by proteas-

es occurs in this hydrophilic module (Aoba *et al*., 1991, 1992a,b). The degraded amelogenin products lacking the C-terminal hydrophilic segment are still sparingly soluble, being stable in the secretory extracellular space (Aoba *et al*., 1987b). One of the crucial steps for the removal of amelogenins is the cleavage of the N-terminal hydrophobic segment, which causes the dramatic change in solubilities of the amelogenin products. Indeed, the amelogenin fragments of lower molecular masses, lacking both N- and C-terminal segments, were found to accumulate in the enamel fluid separated from pig secretory enamel (Aoba *et al*., 1987c). Thus, the accessibility of proteases to the N-terminal cleavage sites is likely a rate-limiting step in the processing and removal of amelogenins.

In connection with the presumed sequential degradation steps of the amelogenins, enamelysin and serine proteases of 76- and 78-kDa molecular weights (Tanabe *et al*., 1992), both of which were concentrated in the outer (youngest) secretory enamel adjacent to the ameloblasts, can specifically cleave the C-terminal segment of the secreted porcine amelogenins as expected, and, more interestingly, these enzymes are not found in the inner (older) secretory enamel, in which EMSP1 (possibly other proteases of 30- to 35-kDa molecular weights) that can cleave the N-terminal segment of the amelogenins exists in place of the high-molecular-weight proteases.

Collectively, an intriguing hypothesis remains to be tested: *i.e.*, that the secreted enamel proteases themselves are processed during amelogenesis, and that their post-secretory degradation, as well as that of the amelogenins and other matrix proteins, might be delayed in fluorotic enamel.

(4) Fluoride Uptake into Developing Enamel: Fluoride Pools in Stable and Labile Forms

Tooth mineral (both enamel and dentin) provides a permanent record of fluoride exposure during the forming stages, because fluoride can be stabilized in the tissue after its incorporation into the lattice sites of apatite crystals (Elliott, 1994). Studies on human fluorotic enamel (Richards *et al*., 1989) further suggest that the fluoride content of erupted fluorotic enamel represents fluoride acquired during tooth formation, and that once tooth formation is completed, further uptake of fluoride prior to eruption may be negligible. The ingested fluoride is absorbed from the gastrointestinal tract and then transported *via* the circulating blood in undissociated or dissociated forms of HF or F^-. The maximum fluoride content of the enamel tissue, on a weight basis, is attained in the early forming stages (Weatherell *et al*., 1975). Studies using enamel organ culture (Bawden *et al*., 1982, 1987; Bawden and Crenshaw, 1984) further demonstrated that fluoride uptake is independent of calcium uptake and is not under direct control by the ameloblasts. Free fluoride concentrations in enamel fluid isolated from pig secretory enamel (Aoba and Moreno, 1987) are in the range of 10^{-6} mol/L. This analytical result supports the concept that the fluoride levels in developing enamel are directly related to plasma fluoride levels (Speirs, 1986). To date, there has been no solid evidence for the binding of fluoride with proteins, at least in the extracellular space (Lussi *et al*., 1988).

Most of the fluoride transported in ionic forms can be readily incorporated into growing enamel crystals, if the precipitation reaction is not down-regulated by inhibitors, such as abundant protein moieties existing during the enamel secretion. However, analytical data of the forming enamel in pigs and rats (Aoba *et al*., 1989a, 1990b) showed that a substantial fraction of the fluoride ions remain in a labile form, which are free in the fluid or associated with organic matter. This labile pool of fluoride corresponds to 25-30% of the total fluoride in the secretory enamel, while the corresponding pool becomes marginal (less than a few % of the total fluoride) in the mature enamel, where most of the matrix proteins have been removed, so that the mineral surface most likely becomes available for reactions with fluoride ions. Under high fluoride exposure, such as in rats after the administration of 100 ppm fluoride in the drinking water for 4 wks (Aoba *et al*., 1990b), it was further confirmed that the total fluoride content of rat enamel increases, and that only a marginal fraction of the total fluoride, even in the secretory enamel, is present in the labile form. This facilitated incorporation of excess fluoride in the stable form is explained by the fluoride-induced acceleration of precipitation kinetics as described below, which may compete well with the inhibitory function of matrix proteins. Currently, the understanding of the kinetics of fluoride uptake into developing enamel has improved substantially, but there is still a lack of knowledge about free fluoride concentrations at the mineralizing sites under various fluoride regimens.

(5) Are the Roles of Fluoride Ions in Developmental Enamel Mineralization Obligatory or Voluntary?

Fluoride is well-known to have unique properties among the varieties of regulators involved in the proliferation and growth of calcium phosphate salts *in vivo* (Aoba, 1997). This ion substitutes for a column hydroxyl in the apatite structure (fluoridation of the lattice), giving rise to a reduction of crystal volume (Elliott, 1994) and a concomitant increase in the structural and chemical stability (or decreasing the thermodynamic solubility) of the resulting apatite crystals (Moreno *et al*., 1974). An important conclusion drawn from crystal growth studies of apatites *in vitro* is that the precipitation kinetics is determined primarily by the degree of supersaturation in solution with respect to the forming crystals (Margolis and Moreno, 1990; Johnsson and Nancollas, 1992). For each calcium phosphate phase, the degree of saturation (DS) can be expressed as the ratio of the ionic activity product (IP) of the ionic lattice constituents (reflecting a given stoichiometry) in solution to the solubility product (Ksp) of the solid, *i.e.*, DS = IP/Ksp. Thus, the well-documented effect of fluoride as an accelerator of apatite precipitation is explained by the lowering effect of Ksp values, depending on the incorporation of fluoride into the precipitating apatite crystals (Varughese and Moreno, 1981).

Under the physiological condition as found in the enamel fluid, hydroxyapatite (or carbonated hydroxyapatite) as a prototype for biomineral is thermodynamically more stable than acidic calcium phosphate salts, such as octacalcium phosphate (OCP), while the precipitation of acidic precursors is kinetically more favorable than apatite precipitation. Accordingly, Brown *et al*. (1987) proposed the theory that apatitic biomineral in enamel (also in hard tissues of mesenchymal origin) is formed through a precursor mechanism in which OCP precipitates first and then hydrolyzes irreversibly *in situ* to a transition product, "OCP hydrolyzate". The unique morphology (*i.e.*, long plates) of formed enamel crystallites provides compelling evidence for the participation of OCP in enamel mineralization. The rapid, dramatic changes in chemical composition taking place in the vicinity of secretory ameloblasts also support OCP as an initially formed mineral during amelogenesis (Aoba

et al., 1998). Collectively, the enamel biosystem achieves the formation of elongated enamel crystals in a well-aligned manner by executing consecutively the two-dimensional growth of an OCP-like precursor and the subsequent epitaxial growth of apatite units on the template (Iijima *et al*., 1992a; Miake *et al*., 1993), concomitant with hydrolysis of the templates themselves (Iijima *et al*., 1992b). This entire enamel mineralization is characterized by gradual growth of thin ribbons, especially their thickening, rather than by proliferation in the number of crystals (see Fig. 1). In this process of enamel crystal formation, fluoride at low concentrations, as determined in the enamel fluid, appears to play a pivotal role in accelerating OCP-apatite conversion and producing ultimately elongated "plate-like" apatite crystals. In previous experiments conducted in media resembling the enamel fluid (in terms of supersaturation level, pH, ionic strength, and temperature), it was verified that, whereas OCP thin ribbons remain stable for considerable periods in the absence of fluoride, the precipitated OCP ribbons are readily hydrolyzed through solid-state transformation (*i.e.*, generating the apatitic lattice structure without alterations in their original morphologies) at levels of 0.05 through 0.4 ppm fluoride (Mura-Galelli *et al*., 1992). In this circumstance, the involvement of fluoride at low concentrations seems to be obligatory in enamel mineralization. It is of paramount interest to know whether normal enamel crystal formation is achievable in the complete absence of fluoride in the mineralizing milieu. But the ubiquitous inclusion of fluoride in foods, soil, and other natural products (Whitford, 1991) would make it very difficult for such experiments to be designed and executed with the use of any conventional animal model.

Another known action of fluoride is to induce the nucleation and growth of apatite crystals, devoid of the participation of acidic precursors, at neutral conditions and physiological temperature; this situation was evident at fluoride levels of 0.5 ppm or higher in the media resembling the enamel fluid (Mura-Galelli *et al*., 1992). Such concentrations are hardly achieved in tissue fluids *in vivo*. However, ultrastructural studies (Yanagisawa *et al*., 1989) have demonstrated apparently *de novo* proliferation of apatite nuclei in the outermost surface regions of very severe cases of human fluorotic enamel, showing that the increasing fluoride uptake may locally enhance the driving force sufficiently to ensure precipitation of fluoridated apatite crystals.

(6) Epidemiology and Pathognomonic Features of Human Enamel Fluorosis

Fluoride has been assigned as the single factor most commonly responsible for causing enamel mottling (Pindborg, 1982). However, either intrinsic and/or extrinsic factors may also cause enamel mottling, although such factors have not been identified in man. In rats, chronic acidosis and hypoxia, independent of the level of fluoride exposure, cause enamel opacities that are histologically similar to enamel fluorosis (Angmar-Månsson and Whitford, 1990; Whitford, 1997). Thus, the differential diagnosis of enamel fluorosis from non-fluorotic enamel defects is critical for accurate assessment of the prevalence of dental fluorosis and its epidemiology (Russell, 1963; Fejerskov *et al*., 1988, 1996; Cutress and Suckling, 1990; Pendrys, 1999). The clinical appearance of milder forms of enamel fluorosis is characterized by narrow white lines following the perikymata, cuspal snowcapping, and a snowflaking appearance that lacks a clear border with unaffected enamel (Dean, 1934; Fejerskov *et al*., 1977, 1988). The corresponding enamel lesion is featured histopathologically as a subsurface hypomineralized lesion covered by a well-mineralized outer enamel surface (Fejerskov *et al*., 1974; Thylstrup and Fejerskov, 1978). Electron microscopy further confirmed that the structural arrangement of the crystals appears normal, but that fluoride affects the forming enamel by causing porosity, *e.g.*, widening gaps between the enamel rods and enlarging intercrystalline spaces in parts of the rod (Fejerskov *et al*., 1975, 1994). With increasing severity, the subsurface enamel all along the tooth becomes increasingly porous, the lesion extends toward the inner enamel, and the fluoride content increases. After eruption, the opaque areas may become stained yellow to dark brown, and the more severe forms are subject to extensive mechanical breakdown of the surface (Baelum *et al*., 1986; Fejerskov *et al*., 1991; Richards *et al*., 1992). It is now widely accepted that pitting and larger surface destructions of enamel are post-eruptive features, not true hypoplasia of the teeth, while attrition and abrasion of the hypomineralized enamel or maybe limited mineral uptake may diminish the intensity of the milder forms of fluorosis (Fejerskov *et al*., 1977, 1990, 1996; Horowitz *et al*., 1984; Cutress and Suckling, 1990).

In relation to the pathogenesis of fluorotic enamel, Eastoe and Fejerskov (1984) first reported that fluorosed human enamel with a Thylstrup-Fejerskov (TF) score of 4-6, while having a total protein content similar to that of normal enamel in quantity, had a relatively high proportion of immature matrix proteins. In contrast, a recent report (Wright *et al*., 1996) of a study of 9 "moderately fluorosed" human teeth (developed in 3.2-ppm-F drinking water area), demonstrated that the protein content of fluorosed enamel was greater than that of normal enamel (mean fluorosed, 0.27%; mean control, 0.11%), whereas the amino acid profiles were similar for fluorosed and normal enamel, and there was no evidence of retention of immature matrix proteins in the sample. The apparent discrepancy between the two reports may be explained by the samples' being stored and handled differently. Moreover, Eastoe and Fejerskov (1984) removed the surface layers to avoid the possibility that the post-eruptive adsorption of exogenous proteins, *e.g.*, from saliva, into the hypomineralized areas could influence the analyses. The fluorosed apatite crystals have stronger adsorption affinities for salivary proteins or other moieties (Moreno *et al*., 1978). Post-eruptive challenges could modify the composition of residual proteins in the porous enamel tissue by "smearing out" the amino acid profiles of endogenous residual matrix components, as well as the appearance and clinical features of the hypomineralized fluorotic enamel, as described above. Currently, quantitative information about proteins left in normal and fluorotic enamel is still limited.

Concerning the risks of fluoride ingestion during enamel formation, it was established that a linear relationship exists between fluoride dose and enamel fluorosis in human populations (Fejerskov *et al*., 1994). This indicates that no threshold value exists below which the effect of fluoride on dental enamel will not be manifest. The severity of the enamel lesion depends on the total fluoride intake, regardless of fluoride source, *e.g.*, ingestion from drinking water or supplements. Thus, for every increase of the dose of 0.01 mg F/kg b.w., an increase in the dental fluorosis community index (Fci) of 0.2, as defined by Dean, can be predicted (Fejerskov *et al*., 1996). In recent years, attention has been given to the individual variation in fluorosis for the same water fluoride intake between and

within populations (Rozier, 1999). Therefore, it is an important task to identify putative factors which, alone or in combination, can make individuals more or less susceptible to the aberrant effect of fluoride. Contributory factors may include composition of diet, bioavailability of fluoride, environmental fluoride, climate, and individual physiological and metabolic factors. Fluoride metabolism has been extensively studied in man (Ekstrand, 1996) and in rodents (Whitford, 1989). Following fluoride ingestion, fluoride is rapidly absorbed into the blood plasma, predominantly in the stomach. The amount and composition of stomach content will therefore significantly influence the degree of fluoride absorption. A variety of variables will influence distribution and elimination of fluoride in the body, including renal function, urinary pH, the fluoride pool in bone, and the rate of bone turnover. These variables can be expected to have a significant influence on fluoride susceptibility within individuals. However, in free-living populations, it is remarkable how little variation in dental fluorosis prevalence and severity exists if these populations have been living under a long-term stable fluoride ingestion level (Manji *et al*., 1986a,b). This is particularly evident in populations in developing countries where the predominant source of fluoride comes from drinking water, whereas in contemporary populations, in, for example, North America and Europe, the extensive use of a variety of topical fluorides, as well as often-unknown contamination of food items with fluoride, will substantially influence the relative amounts of fluoride ingested by individuals. Therefore, it is to be expected that a substantial variation between and among individuals in terms of severity of fluorosis may be observed in such populations at present. Although Cutress and Suckling (1990) have claimed that some uncertainty in clinical diagnosis may make it difficult to establish a cause-and-effect relationship, it seems evident, from these authors' studies as well as from all other studies in which appropriate indices for measuring dental fluorosis have been applied, that, so far, fluoride is the only agent known to cause a gradual increase in enamel porosity with increased dose ingested over a prolonged period of time during tooth development.

(7) Experimental Dental Fluorosis in Animal Models

Our current understanding of the biological mechanisms involved in fluoride-induced lesions of enamel and dentin is based mostly on the results obtained in animal experiments. It has long been argued, however, that the oral doses administered daily to the animals are much higher than the doses encountered in man, or that fluoride-induced changes in the teeth of animals may not be relevant to the human situation, because high fluoride doses, in animals, produce true hypoplastic lesions in addition to hypomineralization, entirely different from human dental fluorosis (Creath *et al*., 1989; Smith *et al*., 1993). In carefully designed experiments involving pigs (Richards *et al*., 1986) and sheep (Suckling *et al*., 1988), however, it was proven that the characteristic subsurface hypomineralization in enamel without hypoplasia, as seen in human enamel fluorosis, can be produced after chronic ingestion of fluoride. Furthermore, the previous experiments with rats (Angmar-Månsson and Whitford, 1982, 1984) and pigs (Richards *et al*., 1985) showed that the plasma fluoride concentrations associated with fluorotic dental lesions in the animals are of the same order of magnitude as those which may occur in man.

For many years, it was believed that the secretory ameloblast was particularly sensitive to fluoride. This assumption was derived from the claim that the fluorotic pits were true hypoplasias, and results in rodents exposed to high fluoride doses seemed to support this assumption (Schour and Smith, 1934; Kruger, 1967; Walton and Eisenmann, 1974). A great conceptual inversion occurred after the observation that enamel fluorosis can be produced by the giving of fluoride to pigs after matrix secretion has ceased (Richards *et al*., 1986). This led to a new concept that any alterations in protein synthesis during secretion are not absolutely necessary for the development of fluorosis. Similar approaches targeting wild roe deer and red deer (Kierdorf *et al*., 1996) also proved that the maturation stage rather than the secretory stage is affected. The important conclusions derived from the previous animal studies are that: (i) systemic effects of fluoride, *e.g.*, disorders in calcium homeostasis, are not necessarily involved in enamel fluorosis (Angmar-Månsson and Whitford, 1984; Andersen *et al*., 1986); and (ii) the increase in severity of dental fluorosis directly reflects an increase in fluoride concentration in the enamel (Richards *et al*., 1985; Speirs, 1986; Angmar-Månsson and Whitford, 1990).

(8) Critical Timing and Steps at which Fluoride Affects the Tooth Organ to Result in Enamel Fluorosis

Knowledge about the ages at which fluoride most affects the unerupted and erupted enamel is of extreme importance for the minimization of risks for the development of fluorosis and the promotion of more judicious use of fluoride in caries prevention (Banting, 1999). Many epidemiologic studies of human dental fluorosis (Ishii and Suckling, 1986; Pendrys and Katz, 1989; Evans and Stamm, 1991a,b; Jackson *et al*., 1999) consistently demonstrated that the permanent anterior teeth, which are of the most important aesthetic concern, are at greatest risk for fluorosis during a two-year period extending through the second and third years of post-natal life. A recent study (Burt *et al*., 2000) provided further evidence that, while an 11-month cessation of water fluoridation had little effect on caries, dental fluorosis is sensitive to even small changes in fluoride exposure from drinking water, and this sensitivity is greater at 1 to 3 years of age than at 4 or 5 years. This is in good agreement with the findings obtained from animal studies showing that the post-secretory stages of enamel formation are most critical for the pathogenesis of fluorotic alterations in the mature enamel. It should be emphasized, however, that the severity of fluorosis apparently increases with prolonged exposure to low doses of fluoride prior to, as well as during, the maturation phase (Larsen *et al*., 1986; Suckling *et al*., 1988; Richards, 1990). This "cumulative action of fluoride" is explained, in part, by the fact that protein degradation already starts in the secretory stage; this early degradation appears to be a common event for most of the matrix proteins identified so far in forming enamel (Aoba *et al*., 1987b; Brookes *et al*., 1995; Smith, 1998). In this sense, it is reasonable to consider that the entire period of amelogenesis may be vulnerable to the fluoride effect, so the risk of fluorosis is best related to the total cumulative fluoride exposure to the developing dentition, rather than to "the most critical" specific periods (Larsen *et al*., 1985; Bardsen, 1999; DenBesten, 1999). It is less apparent whether an effect of fluoride on the stage of enamel matrix secretion, alone, is able to produce changes in enamel similar to those described as dental fluorosis in man (Fejerskov *et al*., 1994).

(9) How Does Exposure to Excess Fluoride Provide Aberrant Effects on Enamel Formation?

As mentioned above, it is established that the hypomineralized alterations of fluorotic enamel are not due to general effects of fluoride on calcium metabolism, or to poisoning effects that depress whole-body metabolism, but are primarily due to *in situ* effects of the ingested fluoride in the local environment. The observations that the fluorosed enamel retains a relatively high proportion of immature matrix proteins, characterized by high proline contents (DenBesten and Crenshaw, 1984; Eastoe and Fejerskov, 1984), support the notion of an incomplete removal of amelogenin proteins under excessive fluoride ingestion during development. Whitford (1997) recently stated that "although several other fluoride-induced effects might be involved in the aetiology of fluorosis, it now appears that inhibition of enzymatic degradation of amelogenins, which may delay their removal from the developing enamel and impair crystal growth, may be of critical importance". In the past, several explanations or hypotheses have been proposed for the fluoride-induced retention of amelogenin-derived fragments (as well as the degraded products of other matrix proteins) in the matured enamel. The postulated fluoride effects are categorized into two groups (Table 1): (i) on intracellular events, including gene expression, synthesis, trafficking and secretion of proteins, resorption and degradation of the once-secreted products; and (ii) on extracellular events constituting multivarious interactions between and among matrix proteins, proteases, crystals, and other fluid constituents, particularly fluoride and calcium ions. On the basis of the currently available data, each of the fluoride effects and possible mechanisms is overviewed concisely in the following paragraphs.

(9.1) Dose-dependent fluoride effects on intracellular events

(9.1.1) Might low chronic fluoride exposure cause aberrant effects on cellular proliferation/differentiation and signal transduction pathways?

Fluoride at millimolar levels in aqueous media has been known to affect multiple enzyme activities in the cytoplasm of diverse cell types, *e.g.*, inhibiting lactate dehydrogenase or activating adenylate cyclase (Hodge and Smith, 1965). However, the relevance of these findings to the effects of low chronic doses of fluoride is questionable, since it is unlikely that adequate cellular levels of fluoride necessary to alter the enzyme activities would be attainable *in vivo* (Kaminsky *et al*., 1990). At present, there is little solid information about the dose-dependent effects of fluoride on the proliferation and differentiation of enamel organ epithelial cells or the fluoride-triggered signal transduction pathway in tooth-forming cells. This is due, in part, to a lack of culture models suitable for the investigation of ameloblastic cell lines. In the research field of bone cell biology, fluoride is regarded as an effective anabolic agent in promoting an increase in bone formation, stimulating bone cell proliferation and activities *in vitro* and *in vivo* (Farley *et al*., 1983; Kleerekoper and Mendlovic, 1993). The molecular mechanisms of the osteogenic action of fluoride have been reported to involve the mitogen-activated protein kinase (MAPK) mitogenic signal transduction pathway. Of interest, the reported mitogenic activity of fluoride is biphasic, mitogenic to bone cells at micromolar doses but inhibiting bone cell proliferation at millimolar levels.

TABLE 1

Postulated Mechanisms by Which Excess Fluoride Uptake May Cause the Delay of Degradation and/or Removal of Matrix Proteins in Forming Enamel

Influence on intracellular events:
- Cell proliferation/differentiation/metabolic activities
- Genetic and/or epigenetic regulation of the composition and properties of matrix proteins
- Cytoplasmic transport and secretion of matrix proteins and proteases
- Cyclic RA/SA modulation and the resorption activities by ameloblasts

Influence on extracellular events:
- Conformation and aggregation of matrix proteins
- Binding of fluoride ion with matrix proteins or proteases
- Protein-crystal interactions
- Processing or lifetime of proteases *in situ*
- Alterations of Ca^{2+}-sensitive proteolytic activities

Furthermore, two possible models have been proposed as to how fluoride affects the signaling pathway: One model implies the inhibition of a unique fluoride-sensitive phosphotyrosine phosphatase in osteoblasts, resulting in a sustained increase in the tyrosine phosphorylation level of the key signaling proteins of the MAPK mitogenic signal transduction pathway (Lau and Baylink, 1998); the other model postulates that fluoride acts in coordination with aluminum to form fluoro-aluminate, AlF_4^-, which activates a pertussis-toxin-sensitive G-protein on bone cell membrane, leading to activation of cytoplasmic protein tyrosine kineses (*e.g.*, Src, Pyk2, and Fak), which in turn stimulates the tyrosine phosphorylation of signaling proteins involved in the MAPK pathway (Caverzasio *et al*., 1998; Susa, 1999). The former mechanism may be specific for bone cells, while the latter may operate universally for various cell types. In fact, a recent report involving rat secretory ameloblasts (Matsuo *et al*., 1998) showed that pertussis-toxin-sensitive G-proteins bound to the rER and Golgi membranes were decreased by NaF treatment, suggesting that fluoro-aluminate may block the protein trafficking from the rER to Golgi apparatus and between the Golgi compartments. In accord with this finding, it was demonstrated that the ingestion of excess fluoride causes alterations in the ultrastructure of rat secretory ameloblasts, *e.g.*, disorganization of Golgi stack and accumulation of transport vesicles, indicative of a disturbance in the intracellular trafficking of the cells. As a cautionary note, these results were usually obtained by the administration of high concentrations of fluoride (chronic ingestion of water including 100 ppm F or a single injection of 2% NaF solution) in the animals. Thus, the current consensus is that the ultrastructure and functions of ameloblastic epithelial cells appear to be affected by the administration of higher fluoride concentrations, such as in millimolar ranges, but it is not certain whether low fluoride levels, relevant to the physiological levels of fluoride found in human serum, really do cause aberrant effects.

(9.1.2) May the expression and secretion of matrix proteins and proteases be altered under the fluoride regime?

Similar to the above discussion regarding the effects of fluoride on the signal transduction pathway in the ameloblastic epithelial cells, there is currently little evidence indicating that fluoride at 1

ppm or lower levels in the extracellular fluid acts directly as a modulator of gene expression responsible for the production of enamel matrix proteins and/or enamel resident proteases. Biochemical analyses of the secreted profiles of enamel matrix proteins in fluoride-ingested rats showed no marked changes in the electrophoretic profiles, protein quantities, or amino acid composition of secretory matrix proteins (DenBesten, 1986; DenBesten and Heffernan, 1989a; Aoba *et al*., 1990b). The fluoride-induced alterations in the quantity of secreted enamel proteases (or their activities assessed on zymography or by various enzyme assays) are also controversial. DenBesten and Heffernan (1989b) previously reported the reduced activity of low-molecular-weight proteases (28 and 33 kDa) in fluorotic rat enamel at the maturation stage, although there is no obvious difference in the activity of other proteases having greater molecular weights between rat fluorosed and control enamel samples. In contrast, a recent report (Gerlach *et al*., 2000) showed that the addition of NaF even at final concentrations of 5 mM and 10 mM did not result in inhibition of the enzymatic activity of the crude matrix extract or the activity of individual enamel enzymes.

(9.1.3) High doses of fluoride affect cyclic modulation of ameloblast morphologies and functions responsible for the removal of degraded enamel proteins

Based on studies of human fluorosed enamel (Fejerskov *et al*., 1974, 1975) and rat enamel (Shinoda, 1975), it was suggested that dental fluorosis could in fact be a result of an impairment of the maturation processes taking place during enamel formation. Fejerskov *et al*. (1977) demonstrated that the extent and distribution of porous areas in fluorosed human enamel demonstrate a strong similarity to those seen at certain stages of enamel maturation during normal human enamel formation. At that time, the authors suggested that this could be a result of a direct effect of fluoride on the complicated cellular processes responsible for the removal of water and protein and the concomitant increasing mineral uptake during maturation. The prominent feature of amelogenesis during the maturation stage is the cyclic modulation of ameloblast morphologies between ruffle-ended (RA) and smooth-ended (SA) types (Josephsen and Fejerskov, 1977). This morphologic modulation certainly accompanies functional alternations, giving rise to changes in the extracellular environment adjacent to the cells. For instance, the previous study with pH indicators (Sasaki *et al*., 1991) revealed that the SA-covered zones along the maturation-stage enamel display a pH approximating 7.2, while the RA-covered zones have a pH of about 6.2. Since the solubility of amelogenins or the optimal activities of proteases are known to be pH-dependent (Shimizu and Fukae, 1983; Bartlett and Simmer, 1999), it is likely that the pH cyclings may be directly related to the control of the kinetics of protein removal from the maturing enamel (Smith *et al*., 1996). On the basis of these unique cyclic patterns, Bawden *et al*. (1995) proposed the concept of zone refinement and further suggested that possible alterations in the role of zone refinement in the enamel maturation zone may serve as the biologic mechanisms responsible for fluorosis. According to their definition, cyclic changes in the physical-chemical environment would favor the repeated partial dissolution and reprecipitation of enamel crystals, resulting in a reduction of impurities from the maturing enamel mineral. Apparently, each cycling yields only marginal alterations in magnitude, so numerous cyclings continuously taking place over a long period are required to accomplish enamel maturation.

Information regarding ameloblast modulation has been obtained mainly with the use of rat incisor enamel, because the continuously erupting tooth model is suitable for an analysis of a full spectrum of cell differentiation and cyclic patterns in a single tooth surface (Josephsen and Fejerskov, 1977; Smith *et al*., 1987, 1996). In studies involving this tooth model, it was documented that the frequencies of modulation between ruffle-ended/smooth-ended ameloblasts (RA/SA) decrease in a dose-dependent manner with increasing levels of chronic fluoride exposure (DenBesten *et al*., 1985), and that this perturbation is reversible. The RA/SA zones return to the normal pattern after the discontinuation of fluoride ingestion (DenBesten and Crenshaw, 1984). More detailed morphometric analysis of fluorotic enamel of rat incisors (Smith *et al*., 1993) proved that: (a) ameloblasts associated with maturing enamel remain ruffle-ended for as much as 30% longer than normal *per* cycle, indicating that fewer total modulation cycles are completed *per* unit time by these ameloblasts; and (b) enamel proteins are lost from the maturing enamel layer at a rate that is about 40% slower than normal. At present, however, little is known about the corresponding properties of RA-SA modulation in larger animal models and man, so it is not possible to predict the relationship between the number of cell cyclings and the resulting "cumulative" effects on enamel maturation. Moreover, there is no solid answer as to how and why ruffle-ended ameloblasts become smooth-ended periodically (Smith, 1998). It is an intriguing hypothesis that ameloblasts can monitor the progress or delay of the extracellular breakdown and/or loss of enamel proteins and respond to extracellular messages by altering intervals of RA/SA transition. In connection with the fluoride-induced delay of ameloblast modulation cycles, fluoride ions by themselves could act to modify the signaling pathway, or messages responsible for the ameloblast modulation might be delivered by other extracellular molecules, such as matrix-derived products or labile Ca ions. The quantities of labile Ca ions appear to be particularly tightly coupled with changes in fluoride concentration in the extracellular space, as discussed below.

(9.2) Fluoride-induced modulation of the kinetics of enzymatic degradation of the matrix proteins in the extracellular environment

If the production and secretion of amelogenins and other proteins may not be affected substantially by low chronic exposure to fluoride, it is conceivable that fluoride ingestion, or pools of the ion transported through the ameloblastic epithelial cells, may affect protease activation or the kinetics of hydrolytic reactions taking place within the extracellular enamel matrix. To date, there is no solid evidence for a direct binding of fluoride ions with either proteases or substrate enamel proteins that would down-regulate protease activities. Alternatively, fluoride action may lead to delays in the rates of protein degradation and removal by two possible mechanisms as discussed below.

(9.2.1) Enhancement of amelogenin adsorption onto fluoridated enamel crystals, which in turn impedes protein-protease accessibility

Amelogenins are known to possess unique solubility properties in solution that give rise to rapid transitions in a reversible manner between monomeric and oligomeric states, depending on the conditions (*e.g*., temperatures, pHs, and ionic strength) (Nikiforuk and Simmons, 1965; Shimizu and Fukae, 1983).

Technological advances such as atomic force microscopy now allow us to visualize the "nanosphere" supramolecular structures *in situ*, which the full-length amelogenins and/or their partially degraded products construct in aqueous media (Fincham *et al*., 1994, 1995). As discussed earlier, the drastic changes in amelogenin solubility in the physiological media occur after cleavage of the N-terminal hydrophobic segment, but the corresponding cleavage sites are likely buried inside the molecular aggregates and gain protection from the access of proteases existing in the fluid phase. In such a situation, the solubility of substrate proteins, or equilibrium between protein molecules in monomeric and aggregated forms, is considered as a rate-limiting step in determination of the kinetics of the enzymatic degradation of amelogenins. Additional protection of the putative cleavage sites against proteases may occur by possible changes in molecular conformation of substrate proteins upon protein-crystal associations (Aoba *et al*., 1989b). In fact, the majority of the secreted enamel proteins, including the intact amelogenins, have adsorption affinity for apatite crystals (Aoba *et al*., 1987a), and, experimentally, the proteins residing on apatite crystals were proven to be more resistant to enzymatic cleavages, as compared with the hydrolytic rates of protein degradation in the absence of crystals (Aoba, 1994; Moradian-Oldak *et al*., 1998; Yamazaki *et al*., 2000). Importantly, increasing fluoride uptake by enamel tissue promotes fluoridation of enamel crystals (*i.e.*, fluoride incorporation into the stable pool), which in turn may enhance the protein-crystal association, resulting in retarding effects on the subsequent crystal growth and removal of protein molecules residing on crystal surfaces. In support of the roles provided by the stable pool of fluoride *in situ*, previous work (Tanabe *et al*., 1988) showed that the adsorption of enamel proteins onto apatitic surfaces increases substantially as a function of the degree of fluoride substitution in the crystalline lattice; the appreciable increasing effects were obtained above X = 0.1 in terms of $Ca_5(PO_4)_3(OH)_{1-X}F_X$. Based on the fluoride profiles of human fluorotic enamel (Richards *et al*., 1989), the fluoride content in the surface-subsurface regions of severe fluorotic enamel appears to be X > 0.1. Notably, crystal growth studies conducted in media simulating enamel fluid (Mura-Galelli *et al*., 1992) also demonstrated that fluoridation of apatite crystals increases steeply, up to 0.25 or higher in terms of the stoichiometric parameter X, in concentration ranges of fluoride lower than 1 ppm.

(9.2.2) Fluoride-dependent modulation of Ca concentrations in the mineralizing milieu, which in turn affects Ca-dependent protease activities responsible for the degradation of enamel proteins

The first clue that Ca-dependent proteases are involved in amelogenesis was provided by the finding that the metal chelator EDTA significantly inhibited the proteolytic activity of extracted enamel proteins (Moe and Birkedal-Hansen, 1979). Previous studies involving an *in vitro* assay (Crenshaw and Bawden, 1984) further proved that enamel protease activities on synthetic substrates are sensitive to Ca^{2+} concentrations at millimolar levels; a half-maximum rate of hydrolysis was attained at 0.09 mM Ca^{2+} ions. The reported effective Ca^{2+} levels are consistent with its free concentration range found in the enamel fluid (Aoba and Moreno, 1987). If the activation cascade, *i.e.*, serine amelogeninase, is activated by metalloproteinases (MMPs), which need Ca^{2+} for their own activation (Overall and Limeback, 1988), and operates *in situ*, it is possible

TABLE 2
Total Contents and Surface Pools of Calcium and Magnesium in Pig and Human Enamel

Sample	Total Content (% wt)		Surface Pools[a]	
	Ca	Mg	Qi_{Ca}	Qi_{Mg}
Pig forming enamel[b]				
secretory stage	25.3	0.28	4.7 (4.8)	39.3 (0.79)
early-maturing stage	31.4	0.33	2.2 (2.9)	52.3 (1.21)
late-maturing stage	36.7	0.18	0.7 (6.9)	6.7 (0.52)
Human mature enamel[c]	37.6	0.22	0.2 (3.4)	4.4 (0.60)

[a] The data are expressed in terms of % fraction of the total ion in enamel. The values in parentheses are expressed in terms of μmol/m².
[b] Pig enamel samples were obtained from embedded permanent incisors of six-month-old animals.
[c] Human enamel was obtained from erupted molars which were extracted for orthodontic reasons.

that the overloaded fluoride interferes, indirectly, with their protease activities by decreasing free Ca^{2+} concentration in the mineralizing milieu. The evidence supporting fluoride-induced fluctuations of Ca concentration in the mineralizing milieu was obtained in animal experiments with rat incisors. These experiments showed that high acute doses of fluoride produce calcio-traumatic responses in the form of, first, hypermineralization and, subsequently, hypomineralization in the affected enamel and dentin (Eisenmann and Yaeger, 1969; Larsen *et al*., 1977; Suga *et al*., 1987). These coupled responses are explained by the sequential events whereby fluoride uptake induces a momentary rapid growth (*i.e.*, hypermineralization reaction), but an accelerated consumption of the common ions for hypermineralization brings about a decrease in the supersaturation level in local extracellular fluids, thereby leading to temporary inhibition or kinetic retardation of subsequent mineralization until the fluid composition (or the supersaturation level) is retrieved by the cell-dependent transport of ions into the extracellular fluid (Fejerskov *et al*., 1996). The results of autoradiographic analysis with ^{45}Ca lead to the following important conclusions: (i) Ca ions can be transported rapidly (within a few minutes) from blood capillaries, through the enamel organ epithelia, to the extracellular space (Reith *et al*., 1984); and, (ii) the quantities of the transported Ca ion are limited by the secretory ameloblasts, while more Ca ions are transported through the maturation ameloblasts (Takano *et al*., 1982; Eisenmann *et al*., 1984; Kawamoto and Shimizu, 1997). As to the mechanism by which the secretory ameloblasts can restrict Ca transport into the extracellular enamel, it was demonstrated that rat secretory ameloblasts are among the richest cells with respect to the quantities of Ca-binding proteins. These proteins likely play roles in the tight regulation of cytoplasmic concentration and vectorial transport of Ca ions (Hubbard, 1996). The analytical results of the enamel fluid isolated from pig secretory enamel also substantiated the view that the Ca supply is a rate-limiting step in early enamel mineralization, because the free concentration of calcium in the fluid is lower by one order of magnitude than that found in circulating blood (Aoba and Moreno, 1987), and, as a result, the fluid composition is maintained close to the saturation level of enamel crystals (Aoba and Moreno, 1992).

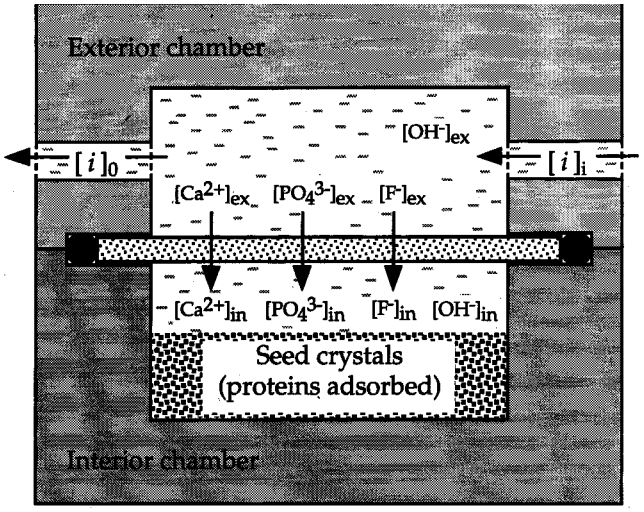

Figure 3. Schematic illustration of experimental set-up utilizing a diffusion chamber, which was developed to assimilate the *in situ* situation as depicted in Fig. 2. The reaction chamber was divided into two (exterior and interior) compartments (each 1.5 mL in volume) by an ultrafiltration membrane (Spectra/por 3, M.W. cut-off 3500). Hydroxyapatite seed crystals, with or without protein coating, were placed in the interior (lower) compartment, which was separated from the exterior compartment by an ultrafiltration membrane. The concentrations of the total PO_4 (3 mM) and NaCl (160 mM) as a background electrolyte were the same between the exterior and interior solutions. The pH value of both solutions was adjusted at 7.3 ± 0.2. Fluoride was added to the solution to yield concentrations of 0.05 to 1.0 ppm, as well as 0 ppm by no addition of NaF. The supersaturated solution contained 1 mM $CaCl_2$ and was delivered at constant rates in the exterior compartment. No calcium was added initially to the experimental solution placed in the interior compartment. In this set-up, Ca^{2+} ions were allowed to diffuse through the membrane into the interior compartment according to the concentration gradient.

In addition to cellular regulation as mentioned above, the regulatory mechanism of Ca^{2+} concentrations within the extracellular mineralizing sites also appears to depend on multiple kinetic and thermodynamic factors involved in enamel mineralization, such as the rate of Ca supply through the cell layers, the rate of Ca consumption for crystal growth, and the solubility of the precipitated solid (Fig. 2). Utilizing a simple crystal growth model as illustrated in Fig. 3, we tested the hypothesis that certain steady-state Ca levels are achieved when Ca supply through the cells (or an artificial dialysis membrane *in vitro*) is the rate-limiting step in the mineralizing system, and that fluoride yields substantial effects on the corresponding Ca level in its concentration-dependent manner (Aoba *et al*., 1995). Key considerations in the experimental design are: (a) the use of a supersaturated solution as predicted in the *in situ* fluid phase; (b) the inclusion of hydroxyapatite seeds with or without precoating by enamel proteins; and (c) the placement of a dialysis membrane to limit Ca flux from the external circulation into the reaction chamber. In practice, changes in the solution composition in the interior chamber, where seed crystals are placed, were monitored analytically as a function of time when the supersaturated solution was fed through the external chamber, which was separated by the membrane. The results obtained showed that: (i) in the absence of seed crystals, the Ca concentrations in either compartment became equal within reasonable equilibration time; but (ii) in the presence of seed crystals, the time course of concentration changes was almost similar, while the plateau levels of Ca concentration in the mineralizing solution stayed lower, in comparison with the level attained in the absence of seed crystals (Fig. 4). Furthermore, fluoride caused decreasing effects on the plateau levels of Ca concentration, depending on the fluoride concentrations added (Fig. 5). The resulting plateau level corresponds to the steady-state condition, which was determined by the difference between the rates of Ca supply and its consumption for precipitation. Since the rate of Ca supply (depending on the Ca concentration gradient between the exterior and interior chambers and the membrane properties) was initially maintained constant in all systems *in vitro*, it is pertinent to conclude that the steady-state levels of Ca concentration were determined primarily by the rate of Ca consumption for precipitation. Notably, the addition of fluoride at low concentrations induced reducing effects on the Ca level in the mineralizing milieu. Obviously, the observed reduction in the plateau level of Ca ion (10^{-4} molar range) cannot be explained by the formation of ion pairs between Ca^{2+} and F^-. The most reasonable interpretation for the fluoride-dependent decrease in the steady-state Ca levels is that the rates of Ca consumption for precipitation are highly sensitive to the increase in fluoride concentration in the range of 1 ppm or lower, and that the precipitation of fluoridated apatite crystals, which have lower solubilities (Moreno *et al*., 1974), allows the Ca^{2+} concentration in the medium in contact with the precipitated crystals to be retained at lower levels. These experimental results also support the concept that the Ca level in local mineralizing sites can be modulated without the disturbance in Ca concentration in the exterior circulation system, as has been proven for fluorotic enamel formation in animals and man. Also, it explains the elegant *in vitro* studies of the effect of fluoride on mineralization of tooth germs (Bronckers *et al*., 1984).

(10) Assessment of the Exchangeable Calcium Pool Located on the Forming Crystal Surface

From the above consideration, it is evident that one of the key factors in both normal and fluorotic enamel formation is the quantities of free or labile Ca ions in the extracellular space. The previous studies with the use of a Ca-chelator, GBHA (glyoxal bis 2-hydroxyanil), demonstrated the appearance of periodic bands on the surface of maturation-stage enamel, indicative of cyclic variations in the amount of Ca ions in labile or loosely bound forms in the developing enamel (Smith *et al*., 1987; Takano *et al*., 1988). The reason why certain fractions of the transported Ca ions can stay in labile or loosely bound forms within the extracellular space is ascribed to the blocking effects of the growth sites by abundant matrix proteins which retard the incorporation of Ca^{2+} (and other ionic constituents) into the lattice positions. To date, however, analytical approaches (McKee *et al*., 1989) failed to provide quantitative information about the corresponding calcium pool or compositional changes associated with the cyclic zones during maturation enamel. Also, the use of radioisotope gives the uptake pattern of ^{45}Ca but does not allow us to differentiate the labile Ca pool from total Ca uptake, due to precipitation or isotopic exchange onto the existing crystals. For assessment of the Ca pool located on crystal surfaces (surface pool, Q_{Ca}), an alternative approach is the use of the competitive adsorption model between Ca^{2+} and Mg^{2+} in solution for the same adsorption sites (Aoba *et al*., 1992a). For instance, our studies on the adsorption of Ca^{2+} and Mg^{2+} onto forming pig enamel and mature human enamel showed that the values of Q_{Ca}, which is expressed as a % frac-

tion of the total Ca, vary significantly with the nature of the enamel mineral, from a 4.7% maximum for the secretory enamel to 0.24% for the late-mature enamel (Aoba *et al.*, 1999). The apparent decrease in Q_{Ca} as a function of enamel development cannot be simply explained by the growth of crystals, or by a reduction in the specific surface area. Indeed, a comparison on the basis of the densities of calcium *per* unit surface area ($\mu mol/m^2$) shows stage-specific fluctuations, *e.g.*, the highest Q_{Ca} at the late-maturation stage. Regarding the state of Mg ions, the values of Q_{Mg} were found to be the highest at the early-maturation stage. The overall results are consistent with the previous findings, showing that the Mg incorporation becomes maximized with the massive removal of matrix proteins during the transitional stage (Kirkham *et al.*, 1988), while the Ca transport, at accelerated rates, continues throughout the maturation stage. In the literature, much attention has been paid to a simultaneous increase in the levels of both magnesium and fluoride in the fluorotic enamel (Robinson *et al.*, 1995), despite the fact that the Mg ion is a strong inhibitor of apatite precipitation, in contrast to fluoride, which is a strong promoter. One may presume that this is due to the formation of magnesium phosphate/fluoride complexes (Robinson, 1997), but a more plausible explanation is that, if excess fluoride causes a reduction in the steady-state Ca concentration in the mineralizing milieu, adsorption of Mg ions for the competitive adsorption sites becomes more favorable, resulting in the increase of Mg content.

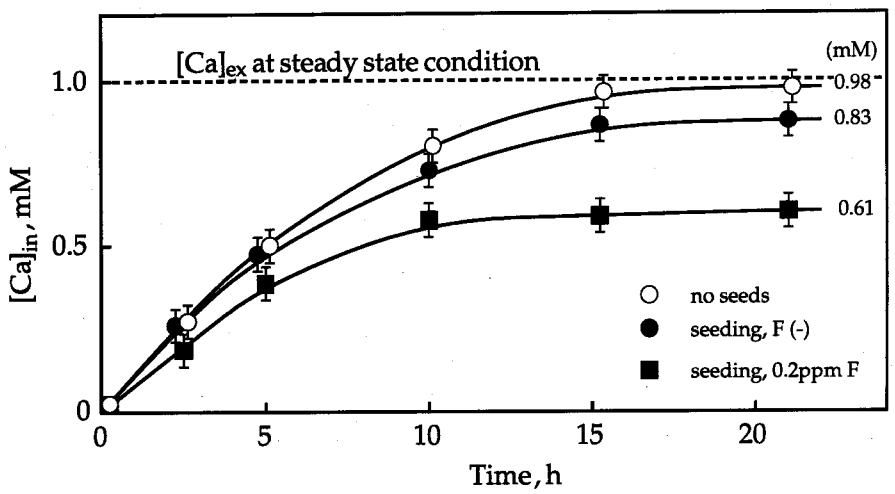

Figure 4. Changes in Ca concentration ($[Ca]_{in}$) in the solution surrounding the seed crystals as a function of time after delivery of the supersaturated solution. Following the Ca transport along the concentration gradient between the exterior and interior compartments ($[Ca]_{ex} > [Ca]_{in}$), a plateau of the $[Ca]_{in}$ was attained after 12 hrs of equilibration. The plateau levels, $[Ca]_s$, are markedly lowered by the presence of seed crystals and the addition of fluoride.

(11) Maximizing Benefits and Avoiding Risks in the Use of Fluoride for Public Health

Ever since the discovery of fluoride's potential as a caries-preventive measure, it has been an ultimate goal in public health dentistry to use fluoride to obtain a maximum caries-preventive effect while at the same time minimizing the risk for the development of dental fluorosis. Water fluoridation is by far the most cost-effective public health measure in caries prevention. It is particularly important to appreciate that, in populations worldwide, access to fluoride-containing toothpaste may be an economic burden of substantial magnitude. As long as it was believed that fluoride exerted its anti-cariogenic effect predominantly by becoming incorporated into the developing crystals of the dental hard tissues, it was obvious that fluoride had to be ingested during tooth formation, because it was believed that the incorporation of fluoride into the forming enamel would significantly reduce enamel solubility during an acid attack. Based on this paradigm, public health dentists argued that fluoride should be ingested during tooth formation. Therefore, early signs of dental fluorosis, which soon became considered as an undesirable side-effect of the beneficial use of fluoride in water, was regarded as merely a cosmetic problem. With today's knowledge about the mechanisms of fluoride action (Larsen, 1975; Fejerskov *et al.*, 1981; Ten Cate and Featherstone, 1996), it is important to appreciate that, as fluoride exerts its predominant effect on the cyclic de- and remineralization processes which take place at the tooth/oral fluid interface, it is possible for maximum caries protection to be obtained without the ingestion of fluorides to any significant extent. Thereby, prevalence and severity of dental fluorosis as a result of caries-preventive programs can certainly be kept at very low levels in all populations today (Clarkson *et al.*, 1996). Dental caries is still a major problem worldwide (Fejerskov and Baelum, 1998), although caries prevalence and severity have dramatically decreased in child populations throughout the world, as already discussed in many reviews (Glass, 1982; von der Fehr and Schwarz, 1994). However, it should be noted that dental caries is not a disease of childhood only, but is a lifelong disease process, and as such, caries incidence remains fairly stable in any population with increasing age (Luan *et al.*, 2000). Moreover, in recent years, it has become

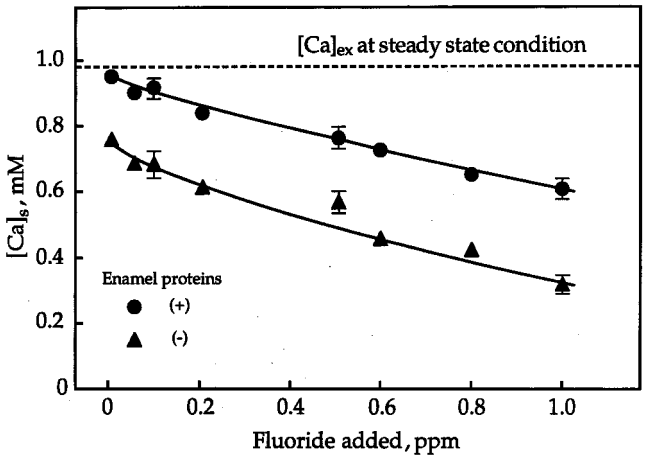

Figure 5. Plots of the steady-state Ca concentration ($[Ca]_s$), which was determined analytically after 30 hrs of equilibration in systems with various fluoride concentrations ($[F]_{ex}$). The $[Ca]_s$ decreased almost linearly when $[F]_{ex}$ was increased in both systems with and without protein coatings of the seed crystals. In both systems, increasing the fluoride concentration resulted in the retention of $[Ca]_s$ at lower levels. The data with deviation bars were obtained from triplicate experiments, while the data without the bars were obtained from duplicate experiments. Note that enamel proteins substantially modified the $[Ca]_s$, by reducing the Ca consumption for precipitation.

apparent that dental caries is still the predominant cause of tooth loss in all populations, and in a modern concept on dental caries (Fejerskov, 1997), it can be fully understood why fluoride, therefore, has to be made available in slightly elevated concentrations in the oral fluids lifelong, to exert its maximum effect on caries lesion development. Fluoride does not, in a true sense, prevent dental caries but rather controls the rate of lesion progression. This has been convincingly demonstrated in studies where the caries-preventive effect of school-based programs with fluoride mouthrinsing or toothbrushing has been studied long after cessation of the programs (Haugejorden *et al*., 1990). These observations are very important, since they highlight the necessity for substantial improvement in our understanding and knowledge about how caries control can be optimized by means of fluorides. There is thus a strong need for sophisticated studies on the association between water fluoride levels and caries experience in large populations of contemporary children. Moreover, detailed studies of the effect of fluoride on carious dissolution *in vivo* under controlled conditions are needed. For example, recently published studies on the effects of fluoridated toothpaste and plaque removal on the development of root-surface caries have showed effects on mineral distribution within exposed root surfaces which cannot be immediately understood (Nyvad *et al*., 1997). Fluoride is still the only agent which has a strong effect on dental caries, and therefore, research efforts should be maximized for a better understanding of how fluoride affects biomineralization processes, both in the oral environment and specially in mineralizing tissues during development.

(12) Concluding Remarks

Enamel fluorosis is caused by the long-term ingestion of fluoride during tooth development. Even low fluoride intake (about 0.03 μg/kg bw) will result in a certain, although low, level of fluorosis in a population. The dose-response relationship is clearly linear, and there is no critical threshold for fluoride intake below which the effect on dental enamel will not be manifest. It is now established that the pathogenic effects of fluoride are not likely due to effects on cellular metabolism and/or systemic metabolism, but rather are due to local effects on the mineralizing environment. In both epidemiological studies of human dentition and experimental studies with animal models, fluoride has been shown to affect predominantly the maturation stage of enamel formation, but analysis of the experimental data also indicates that the severity of enamel fluorosis is related to the dose and the duration of exposure to fluoride. So continuous intake of excess fluoride during and after the secretory stage increases the risks. There is currently no direct proof that fluoride at micromolar levels affects the proliferation and differentiation of enamel organ cells. Importantly, the effects of fluoride on the extracellular events during enamel formation, as well as the cariostatic functions of fluoride, are mostly interpretable on the basis of physico-chemical principles, *i.e.*, increasing the driving force for precipitation of calcium apatites in a free ionic form in fluid phase, stabilizing apatite crystals when the ion is incorporated, and enhancing adsorption affinity of proteins (*e.g.*, enamel matrix proteins or salivary proteins) on the resulting fluoridated enamel crystals. It is likely that fluoride exerts its pathogenic role through alteration of Ca^{2+} activities and thereby activities of Ca-dependent proteases in the fluid surrounding protein-crystal conglomerates in extracellular space, resulting in a delay or arrest of protein degradation and removal from maturing enamel. Certainly, more molecules, currently remaining unidentified, participate in normal enamel formation and in the pathogenesis of enamel fluorosis. Fluoride also affects dentin mineralization throughout life. A recent report (Milan *et al*., 2001) showed that fluoride in the concentration range of 10^{-2} to 10^{-8} M affects casein kinase II and alkaline phosphatase in relation to the decreased phosphorylation observed in dentin phosphoprotein from fluorotic dentin, but much still remains to be elucidated about fluoride's effects on dentin and cementum mineralization. Therefore, an increased understanding of how fluoride affects dental tissues and the biology and chemistry of odontogenesis and cariogenesis is greatly needed before improved guidelines for more cost-effective disease control can be formulated.

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