June 1991

Role of Unerupted Enamel Surface Organic Layer on Fluoride Uptake and Reaction Products Following Topical Fluoride Application

Hashim Nainar

Follow this and additional works at: https://opencommons.uconn.edu/sodm_masters

Recommended Citation
https://opencommons.uconn.edu/sodm_masters/93
ROLE OF UNERUPTED ENAMEL SURFACE ORGANIC LAYER ON
FLUORIDE UPTAKE AND REACTION PRODUCTS
FOLLOWING TOPICAL FLUORIDE APPLICATION

S.M. Hashim Nainar
B.D.S., University of Madras, 1984

A Thesis
Submitted in Partial Fulfillment of the
Requirements for the Degree of
Master of Dental Science
at
The University of Connecticut
1991
ROLE OF UNERUPTED ENAMEL SURFACE ORGANIC LAYER ON FLUORIDE UPTAKE AND REACTION PRODUCTS FOLLOWING TOPICAL FLUORIDE APPLICATION

Presented by
S.M. Hashim Nainar, B.D.S.

Major Adviser
Brian H. Clarkson

Associate Adviser
Huw F. Thomas

Associate Adviser
Norman Tinanoff

Associate Adviser
James Yaeger

The University of Connecticut
1991
Acknowledgements

I would like to thank Dr. Brian Clarkson for being my major adviser. His helpful advice, comments and suggestions made my research rewarding; and he was the mentor that every graduate student hopes for. I would also like to thank Drs. Huw Thomas, Norman Tinanoff and James Yaeger, members of my advisory committee, for their advice and participation during the research and thesis write-up.

I sincerely appreciate the efforts taken by Sandra McCurdy to acquaint me with the laboratory procedures. I am grateful to Dr. Arthur Hand and Christine Pearson for the scanning electron micrographs.

I would like to thank my parents for encouraging me in every possible way and for making me what I am today. My utmost thanks go to my brother for all his support without which I could not have completed the degree. Last but not the least, I appreciate very much the patience shown by my wife during my prolonged absences from home.
## Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgements</td>
<td>iii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>vi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vii</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Review of Literature</td>
<td>2</td>
</tr>
<tr>
<td>i) Caries susceptibility of newly erupted teeth</td>
<td>2</td>
</tr>
<tr>
<td>ii) Preventive effects of topical fluorides on newly erupted teeth</td>
<td>2</td>
</tr>
<tr>
<td>iii) Fluoride uptake in newly erupted enamel</td>
<td>4</td>
</tr>
<tr>
<td>iv) Surface enamel of newly erupted teeth</td>
<td>6</td>
</tr>
<tr>
<td>a) Pre-eruptive enamel maturation</td>
<td>6</td>
</tr>
<tr>
<td>b) Post-eruptive enamel maturation</td>
<td>13</td>
</tr>
<tr>
<td>c) Surface morphology of newly erupted enamel</td>
<td>17</td>
</tr>
<tr>
<td>v) Porous nature of surface enamel of newly erupted teeth and fluoride ion uptake</td>
<td>18</td>
</tr>
<tr>
<td>vi) Membrane effects on fluoride ion uptake in newly erupted enamel</td>
<td>21</td>
</tr>
<tr>
<td>vii) The role of the acquired pellicle on fluoride ion uptake</td>
<td>24</td>
</tr>
<tr>
<td>viii) Primary enamel cuticle (Nasmyth's membrane) on newly erupted teeth</td>
<td>29</td>
</tr>
<tr>
<td>ix) Surface reaction products on enamel following topical fluoride application</td>
<td>32</td>
</tr>
<tr>
<td>a) Chemical assay</td>
<td>33</td>
</tr>
<tr>
<td>b) Morphologic assay</td>
<td>48</td>
</tr>
<tr>
<td>General Objectives</td>
<td>55</td>
</tr>
</tbody>
</table>
List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Experimental Groups</td>
<td>85</td>
</tr>
<tr>
<td>Table 2</td>
<td>Fluoride concentration means and standard deviations</td>
<td>86</td>
</tr>
<tr>
<td>Table 3</td>
<td>Etch depth means and standard deviations (μm)</td>
<td>87</td>
</tr>
<tr>
<td>Table 4</td>
<td>Group coefficients of variance</td>
<td>88</td>
</tr>
<tr>
<td>Table 5</td>
<td>Group ranges</td>
<td>89</td>
</tr>
<tr>
<td>Table 6</td>
<td>Statistical analysis (ANOVA)-Alkali-soluble fluoride (μg)</td>
<td>90</td>
</tr>
<tr>
<td>Table 7</td>
<td>Statistical analysis (ANOVA) - 15 second etch fluoride (ppm)</td>
<td>91</td>
</tr>
<tr>
<td>Table 8</td>
<td>Statistical analysis (ANOVA) - 30 second etch fluoride (ppm)</td>
<td>92</td>
</tr>
<tr>
<td>Table 9</td>
<td>Statistical analysis (ANOVA) - 60 second etch fluoride (ppm)</td>
<td>93</td>
</tr>
<tr>
<td>Table 10</td>
<td>Statistical analysis (ANOVA) - 15 second etch depth (μm)</td>
<td>94</td>
</tr>
<tr>
<td>Table 11</td>
<td>Statistical analysis (ANOVA) - 30 second etch depth (μm)</td>
<td>95</td>
</tr>
<tr>
<td>Table 12</td>
<td>Statistical analysis (ANOVA) - 60 second etch depth (μm)</td>
<td>96</td>
</tr>
<tr>
<td>Table 13</td>
<td>Statistical analysis (ANOVA) - Group Intra-tooth analysis</td>
<td>97</td>
</tr>
</tbody>
</table>
List of Figures

Fig. 1. Unerupted third molar (stored for one day) stained with toluidine blue a) without toothbrushing  b) with toothbrushing  98

Fig. 2. Unerupted third molar (stored for 21 days) stained with toluidine blue a) without deproteinizing  b) with deproteinizing  100

Fig. 3. SEM of unerupted third molar with no surface / fluoride treatment a) without KOH wash (X1000)  b) with KOH wash (X1000)  102

Fig. 4. SEM of unerupted third molar with toothbrushing surface treatment and no fluoride application a) without KOH wash (X1000)  b) with KOH wash (X1000)  104

Fig. 5. SEM of unerupted third molar with deproteinizing surface treatment and no fluoride application a) without KOH wash (X1000)  b) with KOH wash (X1000)  106

Fig. 6. SEM of unerupted third molar with no surface treatment and 2% NaF application a) without KOH wash (X1000)  b) without KOH wash (X5000)  c) with KOH wash (X1000)  108

Fig. 7. SEM of unerupted third molar with toothbrushing surface treatment and 2% NaF application a) without KOH wash (X1000)  b) with KOH wash (X1000)  111

Fig. 8. SEM of unerupted third molar with deproteinizing surface treatment and 2% NaF application a) without KOH wash (X1000)  b) with KOH wash (X1000)  113
Fig. 9. SEM of unerupted third molar with no surface treatment and 1 ppm fluoride application a) without KOH wash (X1000) b) with KOH wash (X1000)

Fig. 10. SEM of unerupted third molar with toothbrushing surface treatment and 1 ppm fluoride application a) without KOH wash (X1000) b) with KOH wash (X1000)

Fig. 11. SEM of unerupted third molar with deproteinizing surface treatment and 1 ppm fluoride application a) without KOH wash (X1000) b) with KOH wash (X1000)
Newly erupted teeth are more susceptible to dental caries. It has been observed that topical fluoride exposure has a greater cariostatic effect on newly erupted teeth than on teeth that have been erupted for some time. Newly erupted teeth have been shown to have organic material on their surfaces. This study investigated the possible role of organic material on enamel surface in fluoride uptake during this period. Specifically, this in vitro study examined the fluoride uptake and reaction products after topical fluoride application on mature but unerupted enamel in the presence or absence of the surface organic layer.
i) Caries susceptibility of newly erupted teeth

Permanent teeth exhibit similar patterns of annual caries attack curves in a community with non-fluoridated water (F<sub>-</sub> < 0.05 ppm) (Carlos and Gittelsohn, 1965). Caries attack reaches a peak from two to four years following eruption with the probability of attack for second molars greatest during the second post-eruptive year, while for most other teeth the maximum attack rate is reached one to two years later. Characteristic of most teeth is the tendency for the attack rate to be relatively low during the first post-eruptive year, to rise rapidly to its maximum value and to begin declining by the fourth or fifth year of exposure (Carlos and Gittelsohn, 1965). Within one year of the mean eruption date, approximately 10% of the permanent first molars were carious whereas the corresponding figure for second molars was over 45%; after this first year, the annual increment for both teeth was approximately 10% (King et al, 1980).

This observation emphasizes the importance of preventive measures on newly erupted teeth.

ii) Preventive effects of topical fluorides on newly erupted teeth

Numerous studies have demonstrated a greater reduction in caries susceptibility of teeth that have erupted during a program of topical fluoride exposure as compared to teeth that were already erupted at the baseline (begining of topical fluoride exposure). This effect has been observed with fluoride applications (Muhler, 1960; Averill et al, 1967; Horowitz and Heifetz, 1969; DePaola and Mellberg, 1973); fluoride dentifrices (Horowitz et al, 1966;


It was suggested that the reduction in dental caries on newly erupted teeth may be because of the more pronounced fluoride uptake in immature enamel of newly erupted teeth than in more mature enamel of teeth that have been present in the mouth for some time (Horowitz and Heifetz, 1969).

In summary, topical fluoride exposure is suggested to have a greater cariostatic effect on newly erupted teeth, particularly when they are susceptible to caries. This effect is noted regardless of whether the subjects consume optimally fluoridated water or not. Frequency of exposure also seems to be a factor. The greater cariostatic effect on newly erupted teeth has been suggested to be due to greater fluoride uptake in immature enamel of
newly erupted teeth than mature enamel of teeth present in the mouth for a longer period.

iii) **Fluoride uptake in newly erupted enamel**

In sound enamel, a fluoride gradient is created during formation and maturation. The fluoride level in sound matured human enamel is in the order of 1000 ppm in the outer 2 μm (Arends and Christoffersen, 1990). Fluoride accumulates in the surface of enamel well before its mineralization is complete. Throughout the entire surface of unerupted or recently erupted teeth, fluoride concentrations are high in the immediate region of the surface and decrease steeply to a relatively low value in the enamel interior (Weatherell et al, 1973) towards a plateau in the middle third of the enamel (Speirs, 1971). The distribution of fluoride concentrations from surface to interior exhibits a "hockey-stick"-shaped power-law curve (Weatherell et al, 1974). Appreciable uptake probably continues for some time after eruption. The situation in the human tooth is more than likely similar to that in newly erupted rat molar enamel, in which the capacity to incorporate fluoride decreases quickly with age. Thus, the optimal time to apply topical fluoride to the tooth surface is soon after eruption when the outer region of the enamel is still fairly permeable. For some time after eruption, the enamel probably remains porous enough to absorb fluoride relatively easily (Weatherell et al, 1977). Joyston-Bechal et al (1971) proposed that the diffusion of ions into enamel is influenced by the volume of water which is loosely bound to the organic material of enamel. Sognnaes (1975) in a review noted that studies using radio-isotope uptake in the dental enamel of rhesus monkeys have shown considerably higher reactivity shortly before and during tooth eruption compared to that in erupted teeth (Sognnaes, 1957). Even when it is
exposed to low levels of fluoride (10 ppm), the enamel of unerupted and erupting teeth does, in fact, react far more intensively than does erupted teeth (Sognnaes, 1965).

Fluoride is deposited a) into the enamel throughout formation, b) into the surface enamel after mineralization has finished but before eruption, and c) post-eruptively at the surface (Brudevold et al, 1956). Younger teeth acquired more enamel fluoride than older teeth (Brudevold et al, 1960). Fluoride can be acquired post-eruptively and this uptake results in an increase of fluoride concentration in the outermost layer of enamel (Speirs, 1959). Fluoride acquisition can occur post-eruptively during the maturation that takes place for a short period after eruption. The reactivity of enamel with fluoride decreases with time after tooth eruption, probably due to post-eruptive maturation (Gron, 1977). Fluoride uptake in enamel from topical fluoride treatment is inversely proportional to the initial fluoride content in enamel (Retief et al, 1983).

Fluoride readily incorporates into the whole thickness of immature enamel but no accumulation takes place in surface enamel. However, as mineralization progresses, fluoride is deposited and concentrated in surface enamel and fluoride given during the late stage of maturation is incorporated into surface enamel (Speirs, 1980). Throughout the life of the tooth, fluoride concentrations are relatively high at the enamel surface. Most of the fluoride in the surface region of enamel in an erupted tooth is acquired prior to eruption, probably during the pre-eruptive maturation period, when the surface of the enamel seems to be porous. For some time after eruption, the enamel probably remains porous enough to absorb fluoride relatively easily.
If mature enamel remains intact, it absorbs fluoride with difficulty and there seems to be relatively little increase in the fluoride concentration of sound enamel surfaces (Weatherell et al, 1977). There is a slower rate of penetration of the outer enamel by fluoride ions. The deeper layers of the enamel which have a low fluoride content take up fluoride more readily than the surface layers which have a high fluoride content (Hardwick and Fremlin, 1959).

In summary, post-eruptive maturation and porosity of the enamel appear crucial in fluoride uptake following topical application in newly erupted teeth.

iv) Surface enamel of newly erupted teeth
a) Pre-eruptive enamel maturation

Enamel when first secreted is soft and translucent, containing on a volume basis, only 10-20% calcium phosphate mineral and 80-90% fluid and matrix protein (Robinson et al, 1981). Transformation of this enamel has been alternatively termed "enamel maturation", "mineralization", or "calcification" (Glick, 1979). Whereas the bulk of the minerals are laid down within a relatively short time, the final accretion of minerals and trace elements requires considerable time, and apparently proceeds throughout the lifespan of the tooth. Maturation appears to be the preferred term for those processes which determine the final composition of the enamel (von der Fehr, 1965).

Glick (1979) reviewed that the increasing mineral volume of enamel is compensated for chiefly by a volumetric loss of water and, to a lesser extent, by the loss of the organic matrix. He cited Diamond and Weinmann (1940)
who suggested that human enamel forms in two distinctly different stages, the first stage representing the incremental deposition of a partially mineralized, acid insoluble organic matrix. The second stage is characterized by sudden loss of the enamel matrix suggestive of a rapid increase in mineral concentration. However, Angmar-Mansson (1971) noted that the withdrawal of organic matter is a continuous process with a linear decrease of organic matter during an equally gradual deposition of mineral salts. Enamel development in human teeth occurs in at least three distinct stages: forming, maturing and mature (Deutsch and Shapira, 1987). Robinson et al (1978) have shown that this pre-eruptive transformation occurs in four fairly discrete stages:
a) partially mineralized enamel is secreted.
b) selective replacement of matrix proteins by tissue fluid begins.
c) almost all the matrix protein is withdrawn and the resulting porosity filled by tissue fluid. The fluid begins, in turn, to be replaced by mineral.
d) the enamel becomes almost fully mineralized, mature and hard.

Suga et al (1987) noted that the maturation stage is not a simple, continuous process, but rather is composed of three sub-stages (phases):
first phase - a secondary increase in mineralization takes place from the surface towards the deeper layer
second phase - then a tertiary increase in mineralization appears to occur from the deeper layer toward the surface
third phase - finally, the narrow outer layer, which has been mineralizing very slowly during the previous phases,
mineralizes quickly and heavily and finally attains the highest degree of mineralization of all the layers. The narrow outer layer shows the lowest degree and slowest rate of mineralization during the first and second phases of maturation (Suga et al, 1987).

Suga et al (1987) in a review noted that the histologic and chemical changes which appear in the enamel organic matrix, accompanied by the secondary increase in mineralization during early maturation are:
a) removal of organic matrix substances due to the degradation of matrix performed by proteolytic enzyme activity during the matrix formation stage and the resorptive function of the ameloblasts; and
b) a new deposition of organic substances (protein and/or mucopolysaccharides) from the ameloblasts into the enamel matrix. Removal of organic matrix substances provides the space for further secondary growth of apatite crystals (Suga et al, 1987).

Glick (1979) reviewed that mineral deposition advances from the earliest formed matrix at the cuspal portion of the enamel-dentin junction and expands peripherally and cervically in a pattern approximating the incremental deposition of the enamel matrix. Enamel adjacent to the enamel-dentin junction rapidly attains a high mineral density. An increasing gradient of mineral concentration also exists from the enamel surface towards the enamel-dentin junction. The enamel comprising the lingual surface appears to mineralize in advance of that of the labial, although at equal distances from both the incisal edge and the enamel-dentin junction, the mineral concentration of both labial and lingual enamel is the same. As the
density of the mineral phase approaches maximum values, the mineral gradient is reversed. Thus, external enamel contains approximately 86% volume mineral with a decrease to between 80-85% in interior regions (Glick, 1979).

The inorganic crystals nucleated in the organic matrix grow mainly at the expense of interstitial fluid, until they finally constitute up to about 86% by volume in normal permanent enamel (Angmar et al, 1963). Since a greater part of the water in fully mineralized enamel is firmly bound to the mineral phase (Carlstrom et al, 1963), very little space is left between the crystallites where water and ions can pass freely (von der Fehr, 1965). Neuman and Neuman (1958) observed that as the mineralization approaches quantitative completion, the deposition of minerals will be progressively slowed down as a result of restricted ionic movement (cited by von der Fehr, 1965).

The amount of protein increases gradually by volume across the secretory stage, reaches a peak early during the maturation stage, and then declines rapidly thereafter (Smith et al, 1989). Robinson et al (1988) noted that in bovine deciduous enamel, protein decreased throughout development from 20-30% to a minimum of 2-3% in mature tissue. Mineral content remained fairly consistent during secretion (15-20%) rising to 70-80% in mature enamel. Water content rose from varying levels to 60-70% at the secretion/maturation boundary decreasing to about 20% in mature enamel (Robinson et al, 1988). Mineral per volume during secretion in human teeth is ~ 27% (Robinson et al, 1981). The mineral component begins to increase in early maturation before a net loss of protein is observed in the developing enamel (Smith et al, 1989).
The principal protein type of the enamel organic matrix are hydrophobic amelogenins which are abundant in the younger matrix (Fincham, 1982). The other type of matrix proteins are the enamelins with an acidic character and appear to be associated with the mineral phase of the enamel (Termine et al, 1979). Both amelogenins and enamelins are found at all stages of enamel development; the proportions of the enamelins are less than those of the amelogenins in the younger matrix, with the enamelins becoming dominant in the more mature enamel matrix (Termine et al, 1980); the protein content of maturing enamel is low, but the proportion of enamelin is high (Deutsch and Alayoff, 1987). The loss of protein from enamel is accompanied by changes in its overall amino-acid composition suggesting a selective loss of certain protein components and retention of certain others, possibly due to their strong interaction with the mineral phase or due to their high insolubility (Deutsch and Alayoff, 1987).

Bai and Warshawsky (1985) concluded that amelogenins are the non-structural, hetero-dispersed particulate material in the inter-crystallite space. They noted that enamelins constitute the integral template protein which initially provides for elongation of enamel crystallites; and then regulates the continuous growth in width and thickness during maturation and are progressively displaced to the periphery. The role of amelogenins in enamel mineralization is not clear, but the fact that these hydrophobic non-polar proteins, which constitute over 90% of enamel matrix proteins, are lost during or just before the stage where secondary mineralization commences, which involves an abrupt increase in calcium and phosphorous accumulation in the developing tissue, might suggest that these proteins are
involved in regulation of crystal growth, perhaps to some extent by inhibition (Deutsch and Alayoff, 1987). Amelogenins may play a regulatory role in controlling the mineralization process by isolating and preventing the crystallites from fusing; this would ensure that elongation and appositional growth in thickness and width occur in a regulated way to produce a homogeneous population of enamel crystallites organized in three dimensions (Bai and Warshawsky, 1985). Deutsch and Alayoff (1987) noted that earlier in 1985, Traub et al had indicated the acidic enamelin proteins in the nucleation of enamel mineral, although their role is not yet understood.

It has been suggested that the primary extracellular protein secreted by the ameloblast is of high molecular weight (65 kD), and that the complex of proteins and polypeptides seen in the developing matrix reflects the progressive degradation of this "pro-enamel" protein (Fincham, 1982). Protease activity within the extracellular matrix has been postulated as the mechanism for matrix protein degradation (Fincham, 1982). In vitro studies revealed that the rate and degree of breakdown of enamel proteins varied markedly, depending on the stage of amelogenesis (Smith et al, 1989). In rat enamel, secretory stage proteins showed slow in vitro degradation with accumulation of proteins near 18 kD; early maturation stage enamel proteins showed more rapid breakdown with little accumulation of proteins near 18 kD, whereas late maturation stage enamel proteins showed complete degradation (Smith et al, 1989).

Aoba et al (1989) observed that coating hydroxyapatite crystals with enamel matrix proteins (from secretory stage) resulted in a retardation of fluoride incorporation into the crystals when exposed to fluoride solutions, as
a result of an inhibition of apatite reprecipitation. They also noted that the growth kinetics of fluoridated apatite onto hydroxyapatite seeds decreased with increasing coverage of the seed surface with enamel proteins (from secretory stage). Degradation of parent amelogenin with the cleavage of specific segments leads to a loss of the adsorption affinity onto apatite crystals and some of the fragments derived from the hydrophobic (sparingly soluble) amelogenins become soluble in the liquid phase (Aoba et al, 1990). Amelogenins having a 26 kD molecular mass displayed the highest adsorption affinity onto the apatite crystals among the amelogenins (Aoba et al, 1990). Lussi et al (1988) noted no in vitro binding of fluoride to isolated organic matrix of secretory bovine enamel. Aoba et al (1987) have shown that originally secreted (parent) amelogenins selectively adsorb onto surface of hydroxyapatite crystals in contrast to the smaller polypeptides derived from degradation of the intact molecule which do not adsorb substantially onto the hydroxyapatite crystals. Robinson et al (1982) noted that there was a tendency for smaller molecular weight material to persist in the maturing enamel after most of the matrix had been withdrawn. They also observed a 55 kD material that persisted in the maturing tissue after most of the matrix had been withdrawn; this material increased dramatically when the enamel was treated with a demineralizing agent. This 55 kD material was released from the mineral when enamel (stripped of enamel organ) was incubated at 37°C in a moist environment; this release was not inhibited by proteolytic inhibitors but was reduced by fluoride ion (Robinson et al, 1982).

In summary, enamel when it erupts demonstrates an outer layer of highly mineralized tissue. Enamel at this stage also has organic matrix proteins, primarily enamelin associated with the mineral phase; and
smaller molecular weight polypeptides (amelogenins) in the liquid phase within enamel.

b) Post-eruptive enamel maturation

Eruption of incompletely mature enamel occurs in human teeth (Crabb, 1976) but is followed by post-eruptive mineral accumulation (Robinson et al, 1988). Upto a depth of about 10 μm, unerupted enamel of third molars was etched significantly more deeply than enamel of erupted third molars and also upto a depth of ~ 10 μm, significantly more fluoride was found in erupted enamel than in unerupted enamel. This difference is mainly attributed to the effect of fluoride from the oral environment on erupted enamel (Grobler and Joubert, 1988). At and shortly after eruption, changes take place in the enamel to adapt the surface layers to the new environment (post-eruptive maturation) (Dirks, 1966). The process of post-eruptive maturation is not completely understood, but it is apparent that it involves changes in both the mineral and organic components of the enamel. Briner and Rosen (1968) suggested that the post-eruptive maturation of dental enamel was governed by two distinct opposing processes: mineralization effected by the uptake of calcium and phosphate from saliva; and demineralization by acid from bacteria. By treating young rats (20-21 days old) with agents known to inhibit bacterial acid production (1% systemic penicillin and topical 1% sodium fluoride solution independently in two different groups), it was found that in three weeks, these agents significantly enhanced the maturation of enamel compared to littermate control rats. On the other hand, germ-free rats treated topically with a 1% sodium fluoride solution showed no significant difference in degree of maturation from similar control rats topically treated with water.
Animal experiments indicate that saliva-tooth interaction is important. The maturation process has been shown to involve mineralization of hypomineralized areas and the disappearance of these areas occurs only under conditions of normal salivation (Speirs, 1967). Although saliva was implicated in post-eruptive mineralization, even when there was deficiency of saliva, maturation could proceed, although retarded (Speirs, 1967).

Electron microscopic observations suggest that maturation involves crystal growth and narrowing of inter-crystalline spaces (Lenz and Newesely, 1965). It was found that the inhibitor of hydroxyapatite crystal growth, disodium ethane-1, hydroxy-1, 1-diphosphonate (EHDP) applied topically to the newly erupted molars of rats, decreased the rate of post-eruptive maturation of molars. On the other hand, fluoride (a known stimulator of hydroxyapatite crystal growth and inhibitor of demineralization) applied simultaneously with EHDP restored the rate of post-eruptive maturation of the molars to that of a normal control rat. Fluoride treatment alone increased the degree of post-eruptive maturation (Briner et al, 1971). In vitro experiments supported the view that restricted access to hypomineralized areas in fissures may be the limiting factor in saliva-induced mineralization, indirectly confirmed by in vitro experiments that mineralization could be induced (Speirs, 1967).

Herrmann and Rozeik (1962) observed that hardness measurements verified that post-eruptive mineralization occurs both in the enamel of rat molars and human teeth (cited by Brudevold et al, 1982). The enamel of older
animals was harder than that of younger animals. The same was true of human enamel, except that it pertained only to the surface layer and not to the body of the enamel (Woltgens, 1986).

It is apparent that saliva-enamel interaction affects primarily the outer portion of the enamel (Hardwick and Fremlin, 1959; von der Fehr, 1965). Penetration of fluoride from oral environment was limited to a depth of about 10 μm by the dense enamel (Grobler and Joubert, 1988). Maturation processes have been demonstrated to take place mainly in the surface layers of the teeth, while sub-surface and deeper areas appear more inaccessible to such changes (von der Fehr, 1965). Several investigators have demonstrated by means of tracer techniques that the surface enamel is more available to physico-chemical reactions than the sub-surface layers. Phosphate analyses of successive layers of enamel indicate that mineralization may continue to a depth of 0.5 mm for some time after eruption. Generally, the P\textsuperscript{32} uptake shows an inverse relationship to the mineral content. In erupted teeth, it has been definitely established that P\textsuperscript{32} in the saliva was the main contributor to the activity in the enamel (von der Fehr, 1965).

Several studies show that the fluoride concentration of surface enamel increases after eruption at a moderate rate which tapers off with age (Aasenden, 1975). The greatest part of post-eruptive maturation takes place during the first year, post-eruptively (Woltgens, 1986). Even though maturation processes may provide only a few percent of the total inorganic content of the enamel, such alterations may drastically influence the amount of loosely bound water and the diffusion of reactants. Furthermore, crystal growth and chemical alterations, due to recrystallization and to heteroionic
exchange, would also contribute to a reduced activity of the enamel (von der Fehr, 1965).

Driessens (1980) observed that there are possibly two apatite phases and a whitlockite phase in tooth enamel mineral with approximate abundances of 10% for the whitlockite, 30% for the Na- and CO$_3^-$ containing apatite and 60% for the slightly carbonated hydroxyapatite (cited by Woltgens et al, 1981). The Na- and CO$_3^-$ containing apatite phase incorporates the Na$^+$ and most of the CO$_3^{2-}$ ions of the mineral (Woltgens, 1986). Both apatites may acquire fluoride ions but apparently the ions occur preferentially in sodium containing slightly carbonated hydroxyapatite, which dominates in the enamel surface where the Ca and P contents are high (Woltgens, 1986). The hydroxyapatite phase is the main phase in the superficial layer between 0-25 μm in depth, while a Na- and CO$_3^-$ containing apatite stays in a nearly constant ratio to the slightly carbonated hydroxyapatite and the whitlockite beyond a depth of 60 μm (Woltgens, 1986). Woltgens et al (1981) reviewed that at a depth of 25-60 μm, the content of a relatively dense apatite phase X is high in young teeth (six months after eruption) which is defective hydroxyapatite - Ca(HPO$_4$)(PO$_4$)$_5$(OH) (Heughebaert and Montel, 1970). During post-eruptive enamel maturation, this phase X is transformed into a Na- and CO$_3^-$ containing apatite (Woltgens, 1986).

On the unerupted and freshly erupted surfaces, the Na and the Mg content varies from 50-80% of the content at 100 μm depth. However, half a year after eruption, the Na and Mg contents were negligibly small at the tooth surface and they remained smaller than in unerupted or freshly erupted teeth upto a depth of 10-30 μm. These data suggest that post-eruptive maturation
occurs in the mineral of the outer layer of the tooth enamel (Driessens et al, 1985). Post-eruptive changes are found most clearly at a depth of approximately 25-35 μm where the Ca and P content decreased with age (Woltgens et al, 1981).

Driessens et al (1985) noted that several studies have shown that especially the Na- and Mg- containing parts of tooth enamel mineral are prone to acid attack and dissolve faster than the other parts of this mineral. This suggests that short intermittent decreases of pH at the tooth surface could be responsible for the extraction of the Na- and Mg- containing parts from the mineral in the outer 10-30 μm. In fact, this depth agrees with the penetration depth expected to occur during pH changes in the fluid of tooth enamel by the consumption of slightly acidic foods and drinks. Penetration depth can be estimated on the basis of our knowledge about the diffusion coefficients for small ions and molecules in tooth enamel. Therefore, these short intermittent decreases of pH are probably responsible for the observed post-eruptive maturation (Driessens et al, 1985).

In summary, post-eruptive enamel maturation occurs with saliva-tooth interaction in the oral environment. This process provides only a small percent of the total inorganic content of the enamel and primarily affects only the surface layer of the enamel.

c) Surface morphology of newly erupted enamel

The pre-eruptive mature enamel (in unerupted human third molars) shows developmental defects including micropores (upto 1 μm in diameter), thin irregular fissures with rounded borders, highly irregular and porous
Tomes' processes pits, and enamel caps and protrusions (Fejerskov et al, 1984). Crater-like focal holes are also observed with a diameter of 10-15 μm and a depth varying from part of a micron to approximately 10 μm (Fejerskov et al, 1984). The borders of the holes are distinctly demarcated and the walls exhibit exposed enamel crystallites in contrast to the surrounding rather smooth enamel surface; the bottom of the holes appear to be squared or triangular in shape (Fejerskov et al, 1984). The focal holes appear restricted to the very surface of the newly erupted enamel as etching of the enamel surface in vivo totally eliminates this feature (Fejerskov et al, 1984).

The developmental defects in the human enamel surface at the time of eruption contains protein of developmental origin (Fejerskov et al, 1984). These developmental defects possibly make the surface enamel highly porous (at the time of eruption) with a variety of established diffusion pathways into the sub-surface enamel (Fejerskov et al, 1984).

v) Porous nature of surface enamel of newly erupted teeth and fluoride ion uptake

The porous nature of surface and sub-surface enamel undergoing the final stages of maturation has been observed on unerupted, partly erupted, and fully-erupted human teeth, using polarized light microscopy, microradiography, and scanning electron microscopy (Fearnhead et al, 1982). The proteins, it was suggested were replaced by water, which in turn was replaced by mineral during the maturation process with the enamel finally becoming non-porous fully mineralized enamel. The stage of porous enamel takes up preferentially extraneous ions, such as fluoride and magnesium (Robinson et al, 1981). Robinson et al (1988) noted that fluoride ion in
developing tissue reflects tissue fluid rather than the mineral or protein phases. They suggested that uptake of extraneous ions (fluoride) into developing enamel may depend on the porosity and hydration state of the tissue; and that a substantial portion of fluoride may be present as free ion in solution rather than bound to mineral or matrix.

Crabb (1976) examined sections from human premolars just prior to their eruption and found consistently a porous outer layer of surface enamel approximately 180-300 μm wide in unerupted teeth wholly covered by bone. By the time of eruption, the average width of porous outer enamel had become reduced to 100-180 μm. The reduction in the width of the outer porous enamel thus seems to be a function of the eruption time rather than of chronologic age. Fejerskov et al (1984) stated that human enamel at the time of eruption is highly porous with a variety of established diffusion pathways into the sub-surface enamel. Based on scanning electron microscopic study, the mature enamel surface in the central part of the tooth is much more porous at the time of eruption than the cervical enamel. The surface porosity appeared to be a result of incomplete final enamel formation, in particular corresponding to the troughs and the cuspal slopes of the crests of each exposed enamel layer (Fejerskov et al, 1984).

Fearnhead et al (1982) stated that if a newly erupted, relatively "porous" enamel surface is bathed in a salivary environment which favors mineralization, it is reasonable to assume that the maturation process will continue until the majority of the accessible pores are filled with mineral. If, on the other hand, the salivary environment does not favor mineralization or if ionic diffusion into the enamel is prevented for some reason, the
porosities in the surface and sub-surface regions will presumably remain. When this surface and sub-surface porosity is extensive, it causes the diffuse white spot lesions of developmental hypomineralized enamel. This condition is more likely to be due to the failure of the final stages of maturation, than to the malfunction of the ameloblasts in their formative phase of activity. However, interference with ameloblast function during the maturation stages of activity might well affect the degradation and removal of some of the amino acid fraction of the amelogenins, and may result in relatively large residues of organic material remaining between the crystals. This could perhaps interfere in some way with crystal growth and packing during the final stages of maturation. In this case, it is conceivable that, even in the presence of an adequate ion population and accessible diffusion pathways, the presence of an organic "contaminant" might retard or arrest further growth of the apatite crystals already present (Fearnhead et al, 1982). The detection of progressively increasing crystallinity of the enamel post-eruptively is highly significant in that it supports the concept that continuing crystal growth is the principal process by which enamel progressively loses its permeability as the micropores become reduced in size and ultimately obliterated (Fearnhead et al, 1982).

The zone of partially mineralized porous enamel has been shown to take up fluoride ions preferentially (Deutsch and Shapira, 1987). Uptake of $^{18}$F by intact human surface enamel involves diffusion with simultaneous chemical reaction. Exposure of enamel of erupted teeth to the oral environment may result in an increase in the number of chemically reactive sites so that the overall rate of uptake of $^{18}$F is proportionally less influenced by the amount of water available for diffusion (Joyston-Bechal et al, 1971).
In summary, surface enamel of newly erupted teeth is porous and incompletely mature. Thus, it is susceptible to topical applications of fluoride. This fluoride uptake may possibly be modified by alteration in the number of chemically reactive sites. Organic films on the enamel surface forming an semi-permeable interface between the enamel and fluoride solution may modify the reactivity of surface enamel.

vi) Membrane effects on fluoride ion uptake in newly erupted enamel

Membrane refers to a material of whatever shape or form which, when used to separate two solutions, restricts the diffusion of both ions and solvent molecules (Waters, 1970). Materials which are permeable to ions and solvent molecules and which possess fixed charges of one sign due to the presence of ionizable groups are known as ion-exchange materials (Waters, 1971). Membranes show selective absorption for ions of opposite sign to those of fixed charges; for ions of the same sign they act as electrostatic screens (Waters, 1970). Cation-exchange materials contain fixed negatively charged and anion-exchangers fixed positively charged groups (Waters, 1971). Cations preferentially diffuse through cation-exchange membranes and anion-exchange membranes show selective absorption for anions (Waters, 1972). The "fixed charge" or Teorell-Meyer-Sievers theory attempts to explain non-ideal ion-exchange behavior (Waters, 1972).

Ions of the same charge as the membrane are prevented by electric repulsion from approaching the spots at the pore walls where the fixed charges of the same sign are located. From sufficiently narrow pores such ions are virtually excluded and the membrane acts as a "membrane of ideal
ionic selectivity. There is a negative correlation between pore diameter and membrane selectivity. Bivalent and polyvalent ions with a charge of the same sign as the membrane are much more restricted in their permeation across the membrane than univalent ions, not only because of their ordinarily larger size but also because of their higher charge which prevents them by electric repulsion from entering pores which are accessible to univalent ions of the same sign (Sollner, 1974). According to the dynamics of membrane systems with the dense, ion-sieve type, highly ion-selective "permselective membranes" and more than one species of permeable critical ions (ions able to exchange freely across the membrane), the exchange of critical ions across the membranes leads to the progressive degradation of the system until the final state, a Donnan membrane equilibrium would be reached (Sollner, 1974). When a solution of an ionized macromolecular substance is separated from another solution by a semi-permeable substance, which arrests the macromolecules but allows free passage to small ions from both sides, the latter are distributed over both solutions in a peculiar way. Under certain circumstances, depending on the net partial charge, a drop of the pH on one side of the membrane can be effected, accompanied by a rise of the pH on the other side. This is referred to as the Donnan membrane effect. Small ions can exchange across the membrane until a state of equilibrium is reached. When however any of the ions concerned are continually removed as a result of some secondary reaction, the transport of ions through the membrane will also be continuous. Thus, under appropriate conditions ionic distribution causing a pronounced change of pH is possible and predictable as the result of membrane equilibria of the type described by Donnan (Bartheld, 1961).
Enamel can behave as an ion-exchange membrane whose characteristics can change with its ionic environment (Waters, 1970). Enamel is permeable to water, small ions and molecules (Hardwick and Fremlin, 1959; Waters, 1970). The ionic selectivity of enamel is due principally to the mineral fraction. A basic requirement of ion-exchange membranes, viz., that they must have a high density of fixed charges, is fulfilled by the mineral fraction of enamel by virtue of its small crystal size (and hence, large surface area per unit mass) and high mineral content (Waters, 1970). With low concentrations of $^{18}$F, diffusion is accompanied by simultaneous chemical reaction (Duckworth and Braden, 1967). Although both hetero-ionic and iso-ionic exchange of fluoride will occur at these concentration levels, only the former could cause a change in the electrochemical state of the structure (Waters, 1972). If a topical fluoride treatment increases the negative fixed charge density, then in the absence of reactions involving dissolution of the hydroxyapatite and subsequent recrystallization of fluorapatite or calcium fluoride, the increase in the negative fixed charge density at the crystal-solution interface along the diffusion paths will set up an electrostatic barrier, reducing the rate of diffusion of other fluoride ions into the structure. The degree of dissociation of the fluoride ions in topically applied solutions may thus determine the amount of fluoride absorbed, as the rate of diffusion of undissociated molecules containing fluoride, providing they are not otherwise restricted by size, will not be influenced by an electrostatic barrier (Waters, 1972).

In summary, the dynamics of surface enamel and fluoride ion uptake is influenced by the concentration of fluoride ions to which enamel is exposed. Zahradnik et al (1976) have shown by in vitro experimentation that
organic films on tooth display ionic permselective properties. Thus, organic films on the enamel surface may modify fluoride ion uptake. Modified fluoride ion uptake may result in the increased formation of either fluorapatite or calcium fluoride. The organic films on newly erupted enamel surface that could possibly act as an semi-permeable interface are the acquired pellicle and the primary enamel cuticle (Nasmyth's membrane).

vii) The role of the acquired pellicle on fluoride ion uptake

The presence of an acquired pellicle on teeth was first reported by Manly (1943). He observed a recurrent, brown structureless, bacteria-free deposit on the teeth of patients who used a non-abrasive (liquid) dentifrice. The acquired pellicle has been defined as an acellular, essentially bacteria-free film which is deposited on the teeth after eruption (Meckel, 1961, 1965). The acquired pellicle is generally believed to be the product of salivary glycoproteins that selectively adsorb onto the enamel surfaces; this structure has been shown to be reformed, if abraded or scraped away (Meckel, 1961, 1965).

Enamel specimens incubated for two days in sterile complex culture medium at either pH 7.2 or 5.0 showed no film on their surfaces. However, specimens incubated for only one hour in filter-sterilized saliva exhibited a uniformly wide (0.01 μm) film covering the enamel surface. The possibility that filtered saliva contains polymers of bacterial origin, however, cannot be ruled out and should be recognized. Saliva in vivo is not free from plaque-forming microorganisms, and formation of the acquired enamel pellicle may result from bacterial synthetic processes, as well as from non-bacterial processes (Tinanoff et al, 1978). Pellicles of salivary origin may be formed
within minutes on cleaned tooth surfaces. These films are thin, clear, and almost invisible. However, with time they undergo a process of "maturation" and change to a thicker, more readily distinguishable and pigmented layer (Schwartz and Massler, 1969). Meckel (1965) classified acquired pellicle into three categories. Two were formed on the enamel surface, the distinction being that one was transparent while the other had taken on an extrinsic stain. The third type was a sub-surface pellicle which was observed by Leach and Saxton (1966) to penetrate the enamel of approximal surfaces in a dendritic manner for several microns. Moreno (1975) reviewed that the thickness of the acquired pellicle varies from 1 μm (Hayward and Armstrong, 1970) to 10 μm (Meckel, 1968).

Surface pellicles could usually be removed with abrasives (Manly, 1943; Meckel, 1965), but were not removed by brushing the teeth with a moistened brush alone (Manly, 1943; Vallotton, 1945). Sub-surface pellicles required the use of a 2% solution of hydrochloric acid for removal (Meckel, 1965). The tooth pellicle is a highly hydrated structure and given its thickness, it should not constitute an effective barrier for ordinary diffusion processes (Moreno, 1975). However, through electrostatic interactions, it is possible that the pellicle may act as a more efficient diffusion barrier for charged particles than expected from the thickness of the structure (Moreno, 1975).

Moreno (1975) and Zahradnik et al (1976) have shown that ionic transport is reduced across pellicles of salivary origin formed on apatite surfaces. By contrast, neutral water fluxes were only marginally affected indicating that salivary pellicles display ionic permselectivity. Thus because of electrical interactions between the ionic diffusing species and charged sites
on the pellicle matrix, the flux rates of the former are significantly hindered (Zahradnik et al, 1976). Furthermore, this retardation in ionic fluxes is more noticeable for divalent ions than for univalent ions; these results are to be expected for a film with a net electrical charge (Zahradnik et al, 1976).

The permselective properties of the salivary pellicle are not apparent at the early stages of formation; pellicles apparently develop permselective properties with increased development times, which modify ionic transport across these membranes (Zahradnik, 1979). He also noted that pellicles seem to inhibit surface precipitation and, thus may favour greater sub-surface penetration by metastable calcifying solutions.

Joyston-Bechal et al (1976) reviewed that it is generally believed that the movement of fluoride into enamel might be hindered by this organic layer and that this assumption seems to stem from the results of a clinical trial (Knutson and Feldman, 1947) in which fluoride applications were not preceded by a thorough prophylaxis. The reduction in caries incidence was compared with the reductions in previous trials in which prophylaxes were performed and found to be lower (Joyston-Bechal et al, 1976). However, the efficacy of fluoride mouthrinsing in reducing caries has been observed and in these studies there was no control over tooth cleaning prior to rinsing with fluoride solutions (Joyston-Bechal et al, 1976).

Artificial salivary pellicles deposited on enamel blocks appeared to reduce the diffusion of fluoride into the enamel (Tinanoff et al, 1975). They also noted that the reduced fluoride uptake in the surface of enamel blocks that were pumiced after the pellicle formation was not significantly different
from the enamel blocks brushed with water after pellicle formation, probably
due to some pellicle not removed by the pumicing. This finding concurs
with Meckel (1965) who believed that the sub-surface portion of the pellicle
might be undisturbed by a dental prophylaxis. In a similar experiment
performed in vivo, there was no difference in enamel fluoride levels after
three days between a group that brushed their teeth before fluoride treatment
and a group that had a pumice prophylaxis before fluoride treatment
(Tinanoff et al, 1974). Two factors may be involved in interpreting the results
of these studies (Tinanoff, 1976). The pellicle may reduce the initial flux of
fluoride into the tooth; yet, it might also retard the fluoride-enamel reaction
products of the topical treatment from diffusing back out of the tooth.
Another factor which might be involved is the reaction of fluoride with
enamel at high and low concentrations (Tinanoff, 1976). Lower
concentrations of fluoride in topical treatment have been found to produce
fluorapatite, whereas high concentrations produce calcium fluoride (Wei et
al, 1974). If the pellicle reduces the initial flux of fluoride into the tooth, it is
possible that a higher ratio of fluorapatite to calcium fluoride is formed
(Tinanoff, 1976).

The mechanism of uptake of $^{18}$F in both control and samples coated
with artificial pellicle shows a linear relationship between $^{18}$F uptake and the
$\tau^{1/2}$ indicating that the mechanism of uptake is diffusion-controlled (Joyston-
Bechal et al, 1976). It seems that the presence of artificial pellicle has no effect
on the mechanism of uptake of $^{18}$F from solutions of 1.2% Na$^{18}$F. Uncoated
enamel samples show a rapid uptake of $^{18}$F initially from 1.2% AP$^{18}$F
solution followed by a slower rate of uptake subsequently. However, the
pellicle coated samples show a linear relationship between $^{18}$F uptake and $\tau^{1/2}$
throughout 100 minutes. This relationship suggests that the mechanism of
\(^{18}\text{F}\) uptake from \(\text{Ap}^{18}\text{F}\) during the first few minutes is altered by the presence
of artificial pellicle, resembling that obtained with solutions of \(\text{Na}^{18}\text{F}\), i.e., it is
also diffusion controlled (Joyston-Bechal et al, 1976). One difference between
the reactions of enamel with neutral and acid 1.2% fluoride solutions is the
increased amount of calcium fluoride formed when the pH is low (Fischer et
al, 1954). It seems that the presence of the pellicle alters the enamel /\(\text{Ap}^{18}\text{F}\)
reaction in some way; it is possible that because the artificial pellicle itself is
adsorbed by the enamel surface, it restricts the adsorption of a fluoride
precipitate (Joyston-Bechal et al, 1976). Consequently, the amount of calcium
fluoride adsorbed by the enamel surface when treated with a solution of
\(\text{AP}^{18}\text{F}\) would be nearer to that adsorbed when \(\text{Na}^{18}\text{F}\) is used and thus have no
apparent effect on the mechanism of \(^{18}\text{F}\) uptake by the enamel (Joyston-

Fluoride binding to the pellicle has been investigated to observe
whether fluoride uptake into the pellicle might reduce the concentration of
fluoride reaching the tooth and to determine whether the pellicle could act as
a fluoride reservoir. No measurable binding of fluoride to artificial pellicles
was observed (Tinanoff et al, 1975).

In summary, acquired pellicle in total or most of it is removed from
enamel surface by a pumice prophylaxis. Acquired pellicle may modify ionic
transport and display ionic permselectivity. The pellicle might alter the
enamel/fluoride interaction with reduced calcium fluoride formation and
adsorption. There is no binding of fluoride to the pellicle. The greater
cariostatic effect of topical fluorides on newly erupted teeth has been noted
even when the applications were made following a thorough prophylaxis which removes the acquired pellicle. This is suggestive of the role of the primary enamel cuticle in fluoride ion uptake in newly erupted teeth.

viii) Primary enamel cuticle (Nasmyth's membrane) on newly erupted teeth

The existence of the primary enamel cuticle (Nasmyth's membrane) and its development is controversial. This review attempts to ascertain from the literature evidence for the existence/non-existence of the primary enamel cuticle without reviewing in detail the controversial embryological origin of the cuticle.

The first description of the primary enamel cuticle with its relationship to the enamel surface is credited to Nasmyth (1839), though earlier in 1837, Berzelius apparently discovered the removal of a membranous structure from teeth immersed in acid.

Chase (1926) reviewed that Nasmyth (1839) described the primary enamel cuticle as fibrous externally, with a peculiar reticulated appearance on its inner surface though Huxley (1853) stated that it consists of a finely wrinkled or reticulated, structureless membrane through which are seen the impressions of the enamel prisms. Some consensus exists on Paul's (1894) description of two distinct layers, an inner clear, thin structureless pellicle which shows impressions of the ends of the enamel prisms, and an outer layer consisting of one or more layers of epithelial cells (Chase, 1926; Turner, 1954). Chase (1926) noted that the markings on the pellicle called "impressions of the ends of enamel prisms" are not indentations, but are remains of detached portions of enamel adherent to the pellicle.
It is generally accepted that the primary enamel cuticle is present on the entire enamel surface of recently erupted teeth (Darling, 1943; Turner, 1954) though it is lost in older teeth due to degeneration and masticatory trauma (Chase, 1926; Darling, 1943; Turner, 1954; Newman, 1973). But Gottlieb (1921) observed that it is not formed over the whole surface of the enamel and was frequently absent (cited by Chase, 1926). Darling (1943) and Hodson (1966) stated that the primary enamel cuticle is found on enamel surfaces not affected by the forces of mastication.

Nasmyth’s membrane is 1-8 μm thick depending on number of cellular layers (Meckel, 1965) though Hodson (1966), Newman (1973), and Palamara et al (1980) described it as an acellular layer. Meckel (1965) noted that portions of the cuticle are apparently calcified. The mineralization seen is partial mineralization without a mineral bridge to the enamel surface and so the primary enamel cuticle must be removed from the enamel surfaces easily (Meckel, 1965). He also noted that retention of this structure on the surface of erupted teeth appears unlikely since brushing with a soft brush and water was sufficient to remove this structure from surgically removed, unerupted teeth. Apparent mineralization of the primary enamel cuticle results from the presence of large plate-like single crystals in the surface enamel and in the lower layer of the cuticle (Palamara et al, 1980).

Newman (1975) stated that the primary enamel cuticle consists of an amorphous, relatively electron-lucent layer of varying thickness with electron-dense inner and outer borders. Transmission electron microscopy disclosed that the cuticle was a distinct entity, and that its superficial and deep
electron-dense borders were more dense surface concentrations of material rather than separate structures. The absence of all but its electron-dense components on demineralized sections suggests that it consists, in part at least, of a calcifiable matrix (Newman, 1975). It is composed mainly of protein (Nyvad et al, 1988) that is resistant to peptic and tryptic digestion and also contains some mucopolysaccharides and protein bound lipids (Newman, 1975). It forms numerous fine projections in the outer few microns of the enamel (Hodson, 1966; Palamara et al, 1980; Nyvad et al, 1988). The presence of a fine dendritic structure in the very surface of the enamel demonstrates that the crystals in the outermost microns of newly erupted surfaces are embedded in a considerable amount of organic material (Nyvad et al, 1988).

The varied descriptions of this developmental surface coating has led Ellen (1989) to suggest that the term cuticle has been used to refer to different structures. He defined the cuticle to be the non-mineralized electron-dense structure (consisting of basal lamina material) frequently found between the epithelium of the dento-gingival junction and the enamel surface. The reduced dental epithelium persists until the tooth erupts, when it is worn off or remnants may persist in localized areas such as occlusal fissures (Ellen, 1989).

In summary, primary enamel cuticle (Nasmyth's membrane) does exist on the enamel surface of newly erupted teeth. However, it is more precise to refer to it as an developmental organic layer as its cellular component (primarily reduced enamel epithelium) has a varied origin and fate, and the remaining component is essentially basal lamina material. This organic layer is intimately related to the surface enamel in a dendritic manner. The layer is
lost due to attrition after tooth eruption but may persist on surfaces not exposed to attrition. The dendritic sub-surface pellicle of Meckel (1965) and Leach and Saxton (1966) may possibly be this persistent embryologic layer as they described it only on surfaces not exposed to attrition.

Therefore, the organic layer on the surface of unerupted mature enamel (just before eruption) consists of the reduced enamel epithelium and a basal lamina material between the reduced enamel epithelium and the enamel surface. Following topical fluoride application, this organic layer might modify the surface reaction products on enamel.

ix) Surface reaction products on enamel following topical fluoride application
[Note: The nature of the alkali-soluble fluoride deposited on the enamel surface following topical fluoride exposure is controversial. It has been called calcium fluoride or calcium fluoride-like material, though the term alkali-soluble fluoride seems more appropriate. In the following review, the terminology used by the individual researchers has been used with the caution that except for the in vitro studies using calcium fluoride crystals, in most other instances, the term alkali-soluble fluoride would have been more appropriate. Even the term loosely bound fluoride is not specific as some researchers refer to the fluoride in solution (within enamel) as loosely bound fluoride while others use the term to describe the layer deposited on the enamel surfaces following topical fluoride exposure].

Duschner et al (1990) noted that the primary reactions of locally interacting fluorides are limited to extremely thin areas of the outermost
surface of enamel (less than 1 μm); to principally a hyperfine outermost layer of only 0.05 μm (demonstrated by electron spectroscopic depth profiles) (Uchtmann and Duschner, 1982). This could be due to the limited depth where enhanced fluoridation occurs in this tissue (White and Nancollas, 1990).

a) Chemical assay

The reaction products of ionic fluoride with apatite include mixtures of fluorapatite, fluorhydroxyapatite and calcium fluoride with increased amounts of calcium fluoride promoted by increased fluoride concentration or decreased pH (White et al, 1988). Usually, ~90% of fluoride reactivity takes place within the first 5-10 minutes of sample treatment (White et al, 1988). In general, significant enhancement of fluoridation of sound enamel requires reaction conditions conducive to calcium fluoride formation (such as those encountered in APF or high-concentration neutral NaF gels) (White and Nancollas, 1990). There is evidence for two, possibly cariostatic, reaction mechanisms of the calcium fluoride-rich layer (Duschner et al, 1990):

a) it acts as a temporary barrier against acid attack, and

b) during acid attack, the formation of calcium fluoride buffers the surface pH to more neutral values.

The three principal forms of fluoride ion reactivity with apatite are (White and Nancollas, 1990):

a) iso-ionic exchange of F for OH⁻ in apatite:

\[ \text{Ca}_{10} \text{(PO}_4 \text{)}_6 \text{OH}_2 + 2\text{F}^- \rightarrow \text{Ca}_{10} \text{(PO}_4 \text{)}_6 \text{F}_2 + 2\text{OH}^- \]

This process readily takes place with some concomitant \( \text{HPO}_4^{2-} \) loss from the surface layer of apatite crystallites (White et al, 1988).
b) crystal growth of fluorapatite from supersaturated solutions:

\[ 10\text{Ca}^{2+} + 6\text{PO}_4^{3-} + 2\text{F}^- \rightarrow \text{Ca}_{10}(\text{PO}_4)_6\text{F}_2 \]

c) apatite dissolution with CaF₂ formation:

\[ \text{Ca}_{10}(\text{PO}_4)_6\text{OH}_2 + 20\text{F}^- \rightarrow 10\text{CaF}_2 + 6\text{PO}_4^{3-} + 2\text{OH}^- \]

Generally reactions (a) and (b) take place with low fluoride levels in solution (such as between 0.01 and 10 ppm F); this fluoride incorporation would be firmly bound since it is part of the apatite structure (White and Nancollas, 1990). As the fluoride concentration in solution is raised, additional chemical reactions begin to dominate the mineral fluoridation process, including significant amounts of calcium fluoride formation (White and Nancollas, 1990). These reactions can be strongly modified by the source of the fluoride (e.g., monofluorophosphate vs. ionic fluoride), co-ions (e.g., stannous), and conditions of fluoride reactivity (e.g., pH, F concentration) (White and Nancollas, 1990). The reaction dynamics of fluoride at the apatite interface are considerably more complex than the simple reactions of ion exchange, crystal growth, and recrystallization of calcium fluoride highlighted in the above equations (White et al, 1988).

pH stat measurements demonstrated the release of OH⁻ during fluoride reactivity with apatites corresponding to ion exchange formation of fluorapatite/fluorhydroxyapatite or dissolution/reprecipitation formation of calcium fluoride (White et al, 1988). Phosphate release into solution accompanied fluoride uptake under all conditions, including regions where ion exchange predominated (White et al, 1988). Overall, the adsorption of fluoride increased with increasing concentration to a plateau level for each given pH. As pH was lowered, the amount of adsorbed fluoride was increased in the plateau regions of the isotherm. A pH drop from 7.0 to pH
4.5 resulted in an average increase of 50% fluoridation in the plateau regions (White et al, 1988). The solution changes of primary note include:

a) increasing fluoride adsorption (uptake) with increased solution fluoride levels
b) the release of base (that is, the necessity for $\text{H}^+$ to neutralize) during fluoride reactivity
c) the release of large amounts of phosphate during adsorption, with extremely low solution calcium levels (White et al, 1988).

Nearly independent from the chemical nature of the fluorides, there are two types of primary reactions (Duschner et al, 1990):

a) partial degradation of the surface and formation of a precipitate containing mainly calcium fluoride. When the pH was low (below 5) and/or the fluoride concentrations were relatively high (above 500 ppm) the area affected was as thin as 0.5 μm.
b) sorption of fluoride at neutral pH and/or low fluoride concentrations, possibly by exchange with apatite hydroxyl ions.

There is evidence that a major portion of the deposited fluoride leaches away relatively fast after application (Arends and Schuthof, 1975). In vitro studies suggest that the fraction lost consists of unreacted fluoride and calcium fluoride and that the retained fluoride is in the form of fluoridated apatite (Caslavska et al, 1975).

The two principal types of fluoride deposited on enamel surface following topical fluoride application are loosely bound (determined by KOH extraction) and firmly bound fluoride (determined by acid etch analysis). The
terms loosely bound and firmly bound fluoride presumably originated from consideration of the solubility of the fluoridated minerals (with calcium fluoride being more soluble than fluorapatite) and from various retention studies of these fluoride products on tooth surfaces (White and Nancollas, 1990). Caslavska et al (1975) reported that in 24 hours, calcium fluoride formed on enamel could be extracted in 1 mol/L \( \text{KOH} \), whereas the apatitically-bound fluoride was not significantly affected. Dijkman and Arends (1988) noted that the amount of fluoride on enamel determined by \( \text{KOH} \) extraction is a measure of the amount of calcium fluoride-like material on the enamel surface.

The most notable aspect of calcium fluoride reactivity is its increased gravimetric solubility relative to that of fluorapatite and fluorhydroxyapatite in fluids simulating the oral environment (White and Nancollas, 1990). This phase is ordinarily undersaturated in saliva or plaque fluids. Thus, calcium fluoride as a reaction product on enamel (apatite) would be expected to dissolve rapidly in the oral environment (White and Nancollas, 1990). Most likely, loosely bound fluoride \textit{in situ} leaches away through the pellicle with an apparent diffusion coefficient of about \( 10^{-6} \, \text{cm}^2.\text{sec}^{-1} \) (Dijkman and Arends, 1988). Calcium fluoride dissolves more readily in water than in saliva (Saxegaard et al, 1988). Exposure to saliva causes formation of a dissolution-limiting layer which consists of surface-adsorbed phosphates and the dissolution rate is continuously reduced with time of exposure to saliva or phosphate buffer (Saxegaard et al, 1988).

Saxegaard and Rolla (1989) showed \textit{in vivo} that after single topical fluoride application to sound enamel, the loss of calcium fluoride was
arrested after 1-2 days at 70% of the original level. They showed a slight increase in the amount of firmly bound fluoride was significant after two days, possibly due to transformation of calcium fluoride on enamel. However, Dijkman and Arends (1988) noted that after one week in situ, the calcium fluoride-like material had been mostly lost either through "chipping away" from the surface or as local dissolution with subsequent leaching of Ca\(^{2+}\) and F\(^{-}\) through the pellicle. It has been demonstrated that the dissolution rate of calcium fluoride in saliva is very low (Saxegaard et al, 1988). Aqueous solutions containing inorganic orthophosphate and pyrophosphate (at concentrations substantially lower than those usually found in oral fluids) considerably inhibit the rate of dissolution of calcium fluoride; this inhibition was pH dependent, being reduced at pH values below 5.0 (Lagerlof et al, 1988).

The solubilization of calcium fluoride in the oral situation is probably more complex than in in vitro studies with phosphate, pyrophosphate and salivary macromolecules (proteins) acting as strong inhibitors of calcium fluoride dissolution (Chander et al, 1982; Lagerlof et al, 1988; Saxegaard et al, 1988) with increased exposure to phosphate or saliva causing a decreasing dissolution rate (Saxegaard et al, 1988). Rykke et al (1989) in an in vitro study demonstrated that calcium fluoride took up small amounts of proteins as compared with hydroxyapatite; however, when the calcium fluoride was pre-treated with a phosphate buffer, pH 6.8, the protein adsorption increased markedly. Chander et al (1982) showed that the calcium fluoride crystal changes the charge from positive to negative on exposure to phosphate ions and this increased the protein-binding capacity of the calcium fluoride.
Calcium fluoride is deposited on enamel after exposure to high concentrations of fluoride (Saxegaard and Rolla, 1988). This has been demonstrated by electron and x-ray diffraction and electron spectroscopy studies of the reaction product (Uchtmann and Duschner, 1982) and its chemical properties such as dissolution in alkali (Caslavska et al, 1975). Availability of calcium ions is probably the limiting factor in the formation of calcium fluoride on enamel during short exposures (Saxegaard and Rolla, 1988). It was shown that calcium phosphates (such as brushite, whitlockite, monetite, and octa-calcium phosphate) reacted willingly with fluoride to form calcium fluoride, whereas well-crystallized hydroxyapatite reacted only sparsely (Rolla, 1988). This in essence means that both incompletely mineralized intact enamel (which contains high amounts of carbonate and whitlockite) and demineralized enamel (which contains brushite, whitlockite, and secondary phosphate) would lend themselves to calcium fluoride formation when exposed to topical fluoride (Rolla, 1988).

Increased time of exposure, increased fluoride concentration, lowered pH of fluoride agent, and pre-treatment with calcium have been shown to be effective means of increasing KOH-soluble (calcium fluoride) and KOH-insoluble fluoride deposition on enamel in vitro (Saxegaard and Rolla, 1988). The time of exposure seemed to be the major factor with the amount of fluoride deposited on sound enamel after exposure for 24 hours to a neutral 0.48 mol/L solution being more than 35 μg/cm² (Saxegaard and Rolla, 1988). The increase in firmly-bound fluoride, compared with untreated sound enamel, was about 6 μg/cm², although this could be an overestimation (Saxegaard and Rolla, 1988). Thus, the amount of fluoride deposited on enamel (alkali-soluble) outranged by far (> 70%) the amounts of fluoride
acquired in the enamel (bound fluoride) (Saxegaard and Rolla, 1988). The chemical composition and solubility product of "calcium fluoride-like" material formed in vivo are not known; the only information available is that the material is "calcium fluoride-like" and the solubility is very low (Arends and Christoffersen, 1990).

Fluoride concentrations in the range from 100-10,000 ppm produce significant amounts of calcium fluoride as a reaction product (White and Nancollas, 1990). Solution chemical analyses suggest that the fluoridation process can be described by ion-exchange/crystal growth of fluorapatite at lower concentrations, while fluoridation at higher concentrations causes the formation of calcium fluoride-like precipitates (White and Nancollas, 1990). The reaction profiles are strongly dependent upon pH, with calcium fluoride formation occurring with fluoride treatment in the range of 500-1000 ppm fluoride at neutral pH, but at only 10-100 ppm fluoride at lower pH (White and Nancollas, 1990).

Fluorapatite is a finite reaction product of enamel/apatite fluoridation with or without calcium fluoride formation (White et al, 1988). It is suggested that calcium fluoride always be considered as a supplement to rather than a substitute for fluorapatite formation (White and Nancollas, 1990). The cariostatic effects of labile fluoride (loosely bound fluoride and fluoride present in plaque and saliva) have been established as also the cariostatic effects of firmly bound fluoride (fluoride incorporated in the structure of the tooth mineral) (Arends and Christoffersen, 1990).
Intra-oral caries model study noted that only KOH-soluble (loosely-bound) fluoride reduced mineral loss and lesion depths significantly (Ogaard et al, 1990) supporting the view that the major effect of topical fluoride treatment is due to the formation of calcium fluoride on the enamel surface or in lesions (Rolla and Saxegaard, 1990; Ogaard et al, 1990). From a clinical standpoint, various topical preparations that form significant amounts of calcium fluoride-like material on the enamel surface have proved effective for the prevention of dental caries; nevertheless, no consistent correlation has been found relating the amount of calcium fluoride formation to cariostatic efficacy (White et al, 1988).

There has been considerable debate concerning the roles of loosely bound (calcium fluoride) and firmly bound (fluorapatite) fluoride for caries prevention (White and Nancollas, 1990). Both fluorapatite and calcium fluoride can provide fluoride to the solution phase to enhance remineralization and retard demineralization of enamel hydroxyapatite crystallites (White and Nancollas, 1990).

Loosely bound fluoride has been suggested to act as a potential "reservoir" or "depot" source of solution fluoride enhancing remineralization and retarding demineralization processes (White and Nancollas, 1990). It is generally agreed that the major effects of fluoride ions towards enhancing crystal growth and retarding dissolution of dental enamel minerals are derived from the contribution of fluoride ion in the solution phase to increased supersaturation or decreased undersaturation (White and Nancollas, 1990). Thus it is pertinent to consider the relative ability of loosely and firmly bound fluoride to serve as reservoir sources for solution fluoride.
In acidic solutions, fluorapatite, fluorhydroxyapatite and calcium fluoride provide significant amounts of fluoride ions to solution (White and Nancollas, 1990); at neutral pH, however, the ambient fluoride level in equilibrium with fluorapatite or fluorhydroxyapatite is expected to be quite small; unlike the reservoir capacity of loosely bound calcium fluoride which would not be significantly influenced by solution pH (neglecting the formation of surface phosphate layer) and could provide a high ambient solution level of fluoride at neutral pH (White and Nancollas, 1990).

Ogaard et al (1990) noted that apatitically-bound fluoride may serve as a reservoir of fluoride for release during cariogenic challenges; however, this effect may be of little clinical significance. The reason for the major cariostatic effect of the KOH-soluble fluoride (calcium fluoride) may be due to a large reservoir of fluoride, compared with the firmly bound fluoride (Ogaard et al, 1990). In the presence of low levels of fluoride in the solution phase, the crystallization of hydroxyapatite is enhanced while the corresponding dissolution is retarded (White and Nancollas, 1990). Fluoride in the bulk fluorapatite or calcium fluoride solid phase has in contrast limited impact on crystal growth or dissolution kinetics (White and Nancollas, 1990).

White et al (1988) hypothesized that in ion exchange during topical fluoridation phosphate is released from the hydroxyapatite surface in the form of surface-desorbing $\text{HPO}_4^{2-}$ ions. Both materials, pure calcium fluoride and "calcium fluoride-like" material, can be very insoluble depending on solution composition, but it does not affect the solubility products (Arends and Christoffersen, 1990). It has been speculated that the formation of surface phases of calcium-phosphate-fluoride might act as a pH-triggered fluoride
source, providing high levels of solution fluoride for suppression of mineral dissolution (White and Nancollas, 1990).

Laboratory experiments have shown that phosphate ions ($\text{HPO}_4^{2-}$) when present in an aqueous phase together with calcium fluoride, adsorb to the surface of the calcium fluoride crystals and reduce the dissolution rate in water by the formation of a solubility-limiting phase (Saxegaard et al, 1988; Rolla and Saxegaard, 1990). Pre-treatment with phosphate caused a significantly reduced dissolution rate of chemically pure calcium fluoride in 1M KOH; with longer exposure to phosphate resulting in further reduction of the dissolution rate in alkali (Saxegaard, 1988). There is a direct correlation between calcium fluoride mobility (solubility) and $\text{HPO}_4^{2-}$ concentration which was not observed for $\text{H}_2\text{PO}_4^-$ and $\text{PO}_4^{3-}$ ions; thus, the active phosphate species appears to be $\text{HPO}_4^{2-}$ ions (White et al, 1988). It is possible that adsorption of phosphate ions to active sites - that is, kinks - on the calcium fluoride crystals alters their dissolution properties, or that a new phase of calcium phosphate may form (Saxegaard et al, 1988).

Extended exposure to saliva can cause formation of a fluorapatite layer on the calcium fluoride crystals restricting their dissolution further (Rolla, 1988). Chander et al (1982) showed that $\text{HPO}_4^{2-}$ ions adsorbed to calcium fluoride may cause formation of a surface layer of fluorapatite onto the calcium fluoride crystals through a dissolution/precipitation reaction. The transformation of calcium fluoride to fluorapatite may be represented by the following reactions (Chander et al, 1982):

a) $10\text{CaF}_2 + 6\text{H}_2\text{PO}_4^- \rightarrow \text{Ca}_{10}(\text{PO}_4)_6\text{F}_2 + 18\text{F}^- + 12\text{H}^+$

b) $10 \text{CaF}_2 + 6\text{HPO}_4^{2-} \rightarrow \text{Ca}_{10}(\text{PO}_4)_6\text{F}_2 + 18\text{F}^- + 6\text{H}^+$
It is reasonable to consider a growth process in which calcium fluoride first dissolves to give calcium and fluoride ions in solution; and subsequently, fluoride, calcium, and phosphate ions form the apatite crystal (Chander et al, 1982). In this dissolution-precipitation mechanism, the dissolution of calcium fluoride is relatively rapid (Chander et al, 1982). They also noted that it is possible that complex ions of the type $[\text{Ca(PO}_4\text{)}_x\text{F}_y(\text{OH})_2]^{n-}$ are involved in the formation of fluorapatite. Such a coated crystal will probably be more stable and release fluoride at lower pH than the metastable calcium fluoride with surface-adsorbed $\text{HPO}_4^{2-}$ ions (Rolla, 1988). Fluorapatite-covered calcium fluoride crystals are probably not alkali-soluble (Rolla, 1988).

The dissolution-limiting phase which forms on calcium fluoride exposed to saliva dissolves at pH 5.0 or below leading to a slow dissolution of calcium fluoride (Rolla, 1988). This is most likely due to reduced concentrations of $\text{HPO}_4^{2-}$ ions in the environment when pH is lowered and subsequent release of the dissolution-limiting ions from the surface of the calcium fluoride (Chander et al, 1982; Rolla and Saxegaard, 1990). The abundant amounts of phosphate present in saliva are able to inhibit the dissolution rate of even relatively soluble "calcium fluoride" formed at neutral pH (Saxegaard and Rolla, 1988). Presently, the chemical composition and solubility product of "calcium fluoride-like" material formed in vivo are not known. The only information available is that the material is "calcium fluoride-like" and the solubility is very low (Arends and Christoffersen, 1990).

During topical fluoride treatment, the amount of firmly-bound fluoride is also increased to some extent (Saxegaard and Rolla, 1988). It has been suggested that even this alkali-insoluble fluoride may in fact be calcium
fluoride protected by fluorapatite (Chander et al, 1982; Saxegaard and Rolla, 1988). Bound fluoride in the form of fluorapatite or fluorhydroxyapatite is only minimally released under neutral pH conditions; as a result, the greatest influence of firmly bound fluoride is on the acid resistance of mineral phases to dissolution under acidogenic conditions (White and Nancollas, 1990).

A high total level of fluoride in enamel does not guarantee protection against caries (Beltran and Burt, 1988; Arends and Christoffersen, 1990). In situ studies using shark enamel consisting of nearly pure fluorapatite (30,000 ppm fluoride) have shown that caries develops in the enamel under plaque, despite the very large amounts of fluoride present in the shark enamel (Ogaard et al, 1988). It thus appears conceivable that the presence or formation of fluorapatite or fluorhydroxyapatite is not a major aspect of the cariostatic mechanism of fluoride (Rolla, 1988). More important is the recrystallization reaction per se occurring when the environment is supersaturated with fluorhydroxyapatite or fluorapatite when the components of demineralized enamel are immobilized by fluoride ions instead of being lost to the surrounding liquid phase (Rolla, 1988). If this is the case, the fluorapatite formed would be the result of a caries inhibition and not its cause (Rolla, 1988). Thus calcium fluoride is essential in the caries prevention that occurs as a result of topical fluoride application (Rolla, 1988). However, "calcium fluoride" formed under different conditions - that is, high or low pH - may well have different properties, dependent on presence or absence of $\text{HPO}_4^{2-}$ ions during its formation (Rolla, 1988).

Arends et al (1983) showed, in an in vitro demineralization study at pH 4.5 that bovine enamel demineralizes without fluoride in the outer solution
surrounding enamel. However, this demineralization could be inhibited at this pH with fluoride concentration of 30 ppm in the outer solution surrounding the enamel. This leads to the conclusion that a major part of the fluoride present in the solid enamel is not active in caries prevention (Arends and Christoffersen, 1990). In the presence of low levels of fluoride in the solution phase, the crystallization of hydroxyapatite is enhanced, while the corresponding dissolution is retarded (Arends and Christoffersen, 1990; White and Nancollas, 1990). The level of fluoride in solution necessary to inhibit mineral loss vary from 0.1 to 30 ppm depending on the conditions (pH, degree of saturation, fluoride level in solution within enamel, etc.) (Arends and Christoffersen, 1990). Therefore they concluded that fluoride, when present in solution in concentrations around 1 ppm, effectively prevents mineral loss from enamel; the exact level of protection depends strongly on the conditions of pH, etc.

Most likely there is a dynamic equilibrium between fluoride in solution (within enamel) and adsorbed fluoride (on enamel crystallites) at the crystal surface interface (Arends and Christoffersen, 1990). When the crystallite is completely covered by adsorbed fluoride, there is a maximum inhibition of dissolution (Arends and Christoffersen, 1990). Adsorbed fluoride (on enamel crystallites) is mainly responsible for caries prevention in enamel as far as mineral loss and also remineralization is concerned (Arends and Christoffersen, 1990). The level of adsorbed fluoride (on enamel crystallites) necessary for strong inhibition of enamel demineralization in vitro is estimated to correspond to a fluoride concentration in the liquid phase (within enamel) of 1 ppm (Arends and Christoffersen, 1990). The concentration of fluoride in the liquid phase (within enamel) needed in vivo
may be substantially greater whereas adsorbed fluoride (on enamel crystallites) has a maximum value of the order 2 μmol/m², corresponding to the concentration of hydroxyl sites in the surface of the enamel crystallites. If, due to a high fluoride concentration in the liquid phase (within enamel), adsorbed fluoride (on enamel crystallites) is at its maximum value, further increase of fluoride concentration in the liquid phase (within enamel) does not lead to more fluoride adsorption, and crystal growth becomes important (Arends and Christoffersen, 1990).

Arends and Christoffersen (1990) reviewed that crystal growth processes may require high values of fluoride in the liquid phase (within enamel) in order to form fluorapatite and hydroxyapatite. They cited that Christoffersen et al (1984) had observed that the amount of fluoride adsorbed to mineral (enamel crystallites) decreases with increasing pH. The decrease is, however, less than expected for a simple one-to-one exchange of hydroxyl ions for fluoride (in the liquid phase within enamel) in the crystal surface indicating that the adsorption process of fluoride (on enamel crystallites) is more complex than a simple ion exchange. The adsorption results can be described by a model in which a dynamic equilibrium exists between fluoride in solution (within enamel) and the crystal surface. This implies that when fluoride ions are not adsorbed in a given region on the crystal surface, the crystals can dissolve locally (Arends and Christoffersen, 1990).

A model implies that fluoride adsorbed (on enamel crystallites) in a thin monomolecular layer surrounding the enamel crystallites inhibits dissolution; and partly transforms the crystallite surface to fluorapatite (Arends and Christoffersen, 1990). The value of adsorbed fluoride (on enamel
crystallites) can be high for at least two reasons (Arends and Christoffersen, 1990):

a) fluoride in the liquid phase (within enamel) is high due to recent fluoride influx. The influx might be due to direct influx from fluoride in the surrounding outer solution or from fluoride released by, e.g., "calcium fluoride-like" material.

b) fluoride in the solid enamel (within crystallites) is high, and part of the crystallite dissolves. The real crystallite surface decreases, and less fluoride is needed to cover the new smaller surface.

Phenomenologically, fluoride released by the crystallite enters the liquid phase (within enamel) and causes fluoride in the liquid phase (within enamel) and thereby adsorbed fluoride (on enamel crystallites) to increase (Arends and Christoffersen, 1990).

Assuming a Langmuir adsorption, we have

\[ K = \frac{F_a}{(F_{OH} - F_a)F_L} = X/(1 - X)F_L \]

in which

K - adsorption constant
F_a - fluoride adsorbed to enamel crystallites
F_{OH} - concentration of adsorption sites, mol/m^2
F_L - fluoride present within enamel in solution
X - fraction of sites occupied by fluoride ions

Solving the adsorption equation gives

\[ X = \frac{F_a}{F_{OH}} = KF_L (1 + KF_L) \]

For low values of X, we have \( X = KF_L \). The degree of surface covering, X, can also be applied during an acid attack as long as the rate of fluoride adsorption is fast compared with demineralization rate (Arends and Christoffersen,
The rate constant for fluoride adsorption has been estimated to be of the order $2 \times 10^4$ s$^{-1}$m$^{-2}$ around pH 6 (Christoffersen et al, 1984 cited by Arends and Christoffersen, 1990).

Fluoride released from "calcium fluoride-like" in vivo formed material can be as effective as adsorbed fluoride (on enamel crystallites) depending on conditions and amount available (Arends and Christoffersen, 1990). In vivo formed "calcium fluoride-like" material can in principle contribute to adsorbed fluoride (on enamel crystallites); however, the value of fluoride in the liquid phase (within enamel) and therefore the adsorbed fluoride (on enamel crystallites) resulting from this loosely bound fluoride is not known (Arends and Christoffersen, 1990). "Calcium fluoride-like" material could dissolve during a caries attack and provide fluoride in the form of adsorbed fluoride (on enamel crystallites) (Arends and Christoffersen, 1990). The fluoride localized in the inner part of the crystallites is relatively unimportant and latent (not effective) until exposed due to crystallite dissolution (Arends and Christoffersen, 1990).

b) Morphologic assay

The morphologic appearance of the deposits on the enamel surface after topical exposure to fluoride is complicated with calcium fluoride formed consisting of 4- to 15- nm diameter micro-crystallites along with an amorphous matrix of unknown composition among the micro-crystals (Nelson et al, 1984). Large apatite-like crystals, approximately 1 μm in length, were also observed in the outer surface coating following topical fluoride application (Nelson et al, 1984). NMR analysis showed fluorapatite or fluorhydroxyapatite as a reaction product of fluoride uptake under all
conditions, regardless of whether calcium fluoride was formed, unambiguously demonstrating calcium fluoride as an additive rather than substitute form of fluoride reactivity (White et al, 1988).

NMR results indicate that increased fluoridation at lower pH is due to increased dissolution/reprecipitation of fluorapatite rather than the formation of calcium fluoride (White et al, 1988). They noted that at pH 4.5 and higher fluoride concentrations, increased calcium fluoride formation occurs through dissolution/reprecipitation which supported Nelson and Higuchi’s (1970) observations that calcium fluoride formation at the apatite surface should theoretically be preceded by an fluorapatite layer migrating inward within the treated crystals (White et al, 1988).

Electron spectroscopy analyses support the contention that the treatment of enamel surfaces with an acidulated fluoride solution results in the formation of a layer of globules of a calcium fluoride-like material (Rykke et al, 1989). Electron spectroscopy analyses demonstrated that in vitro fluoride treatment (with acidic solutions - pH 4.0 and 5.0) of surface enamel (in bovine incisors) results in calcium fluoride as the main reaction product precipitating on surface enamel and in prism pits; the nearly neutral application (pH 6.0) had very little effect on the composition of surface enamel (Duschner and Uchtmann, 1988). Nelson et al (1983) showed that the firmly bound fluoride underneath the calcium fluoride layer consisted of fluoride-rich surface layers on the enamel crystallites (Rolla, 1988).

Scanning electron microscopy showed globular deposits of calcium fluoride-like material on enamel surface after one hour topical application of
a 2% NaF solution at neutral pH (Cruz and Rolla, 1991). At neutral pH 7 for fluoride incorporation, NMR spectra showed the presence of predominantly fluorapatite, with negligible amounts of calcium fluoride (White et al, 1988). Scanning electron microscopy showed that calcium fluoride-like material formed on enamel by treatment with 2% NaF solution had higher stability in saliva than in water after three weeks incubation (Saxegaard et al, 1988). The calcium fluoride-like globules formed on enamel surfaces following topical fluoride treatment are dissolved in KOH solution (Dijkman and Arends, 1988).

Topical application (four minutes) of NaF solutions (250, 1000, 10,000 ppm F) at pH values below 6.0, produced globular-shaped precipitates on enamel (Barbakow et al, 1983); the globules formed after five minutes of topical application were not removed after 24 hour exposure to the oral environment (in vivo) (Hattab et al, 1988). Neutral sodium fluoride gel drops (0.5% F; five minutes) produced a dense surface coating consisting of calcium fluoride-like globules of about 0.5 \( \mu \text{m} \) in diameter (observed after 24 hour in vivo) (Hattab et al, 1988). At pH values 4.0 and 6.0, the globules increased in number with increasing fluoride concentration (Barbakow et al, 1983). The sizes of the globuli formed after treatment with sodium fluoride solutions at pH 4.0 were inversely related to the fluoride concentration (Barbakow et al, 1983).

Barbakow et al (1984) observed that topical treatment with sodium fluoride solutions (pH 4.0) at varying fluoride concentrations (250, 1000, 10,000 ppm F) for four minutes resulted in the formation of globuli on the enamel surface. They categorized these globuli as smaller or larger than 0.5 \( \mu \text{m} \).
Unwashed specimens that had been treated with the fluoride solutions showed both small and large globuli with predominance of small globuli. Large globuli were more readily removed than the small globuli by washing in water or 1M KOH. All the globuli were removed from the enamel by 1M KOH wash for 24 hours (Barbakow et al, 1984).

The globules formed following topical fluoride exposure ranged in sizes from 0.2-1.0 μm and they appeared to coalesce and form a surface layer microstructure (Nelson et al, 1983). The spherical globules themselves appeared to be agglomerates of still smaller particles approximately 30 nm in diameter; however, the surface coating was not entirely covered with globules and regions of the surface coating (viewed at X 22,000 magnification) consisted of densely-packed particles 30-50 nm in diameter (Nelson et al, 1983). These small particles were presumably similar to the particles which agglomerate to form the 0.2-1.0 μm spherical globules (Nelson et al, 1983). None of the small particles or globules appeared to have a crystallographic morphology (Nelson et al, 1983).

Saxegaard and Rolla (1988) noted that the morphologic appearance in scanning electron micrographs (X 5,200-5,700 magnification) revealed relatively few scattered globules on the enamel surface after single five minute treatment with neutral 0.48 mol/l sodium fluoride solution. The density of the globules increased after one hour and twelve hour treatments. The surface deposits seen after twentyfour hour treatment were not as densely packed as after twelve hours, but appeared larger (upto 1 μm in diameter) with a more cubical shape. They also observed that the density of the globular deposits on the surface increased with increasing fluoride
concentration. The appearance at X 5,000 magnification was quite uniform and relatively smooth after single five minute topical application of 0.48 mol/l sodium fluoride solutions at pH 5.5, 4.5 and 3.5; however, treated surfaces demonstrated microglobular surface deposits at X 40,000 magnification. The globules observed at neutral pH 7.0 were larger but less numerous at X 40,000 magnification (Saxegaard and Rolla, 1988).

Arends and Christoffersen (1990) call the globules "calcium fluoride-like" instead of calcium fluoride because most likely the material in vivo is highly contaminated by phosphate, proteins and possibly other compounds. They reviewed that Christoffersen et al (1988) had observed that the deposits do not consist of chemically pure calcium fluoride, but of calcium fluoride and co-precipitated phosphate ions (HPO$_4^{2-}$). Dijkman and Arends (1988) showed that if "calcium fluoride-like" material is formed in vivo by fluoridated varnish action, the globular structures are still observable after three months, indicating that fluoride leaches away extremely slowly through the pellicle.

"Calcium fluoride" with relatively high concentration of phosphate ions is deposited at neutral pH following topical fluoride application (Rolla and Saxegaard, 1990). These crystals appear to be more soluble than chemically pure calcium fluoride, and also more soluble than "calcium fluoride" formed at lower pH, which presumably contains less phosphates due to less interaction and inhibition by HPO$_4^{2-}$ ions (Rolla and Saxegaard, 1990).
White et al (1988) noted that in 1983, Yesinowski et al had demonstrated the capacity of the $^{19}$F Magic Angle Spinning NMR method to distinguish among fluorapatite, fluorhydroxyapatite, and calcium fluoride both in the bulk phase and on hydroxyapatite surfaces by using the technique of magic angle spinning coupled with the Hahn spin echo method. Powdered enamel demonstrated fluoride uptake behavior similar to that of synthetic apatite, the resulting reaction products differed as analyzed by $^{19}$F MAS-NMR (White et al, 1988). $^{19}$F MAS-NMR showed that the reaction of fluoride with enamel resulted in the formation of an unidentified species with an isotropic $^{19}$F chemical shift in addition to peaks possibly due to apatite fluoride (White et al, 1988). Fluoride treatment of carbonated apatites yielded $^{19}$F MAS-NMR spectra similar to those of the pure synthetic apatites, thus ruling out the carbonate content as the source of the differences observed for enamel. This difference was also observed in treated enamel samples that had undergone a treatment to remove the organic matrix (White et al, 1988).

The highly specific $^{19}$F MAS-NMR method noted that fluorapatite-fluorhydroxyapatite formation accompanied reactions in all regions of the uptake isotherms, including those in which significant calcium fluoride formation occurred (White et al, 1988). Fluorapatite was always formed within a layer of the apatite crystal and calcium fluoride was then deposited with or on this fluorapatite (White et al, 1988). Crystalline fluorapatite precipitates formed at low pH indicating considerable recrystallization (White et al, 1988). Thus loosely bound fluoride cannot be considered as a substitute for, but only as a supplement to fluorapatite formation (White and Nancollas, 1990).
Cruz and Rolla (1991) demonstrated by chemical and scanning electron microscopy analysis the incapacity of an acquired pellicle to interfere with the deposition of alkali-soluble fluoride on enamel in vitro following a single topical application of 2% NaF solution at neutral pH.

In summary, the surface reaction products on enamel following topical fluoride exposure are of two types: alkali-soluble and alkali-insoluble. The primary reaction product appears to be the alkali-soluble fluoride which is suggested to be calcium fluoride or calcium fluoride-like material. This material has low solubility in the oral environment.
General Objectives

This study was undertaken to examine the fluoride uptake and reaction products after topical fluoride application on mature but unerupted enamel in the presence or absence of the surface organic layer. Literature has noted the greater cariostatic effects of topical fluorides on newly erupted teeth but no study has examined this phenomenon in a definitive manner. It has been suggested that this effect is because of the greater fluoride uptake in immature enamel of newly erupted teeth. It has also been suggested that this greater fluoride uptake is caused by the process of posteruptive maturation and porosity of immature enamel. However, the fluoride uptake may also be modified by organic films on the enamel surface. It has been shown that acquired pellicle can modify ionic transport and display ionic permselectivity. Thus after topical fluoride application, the pellicle might alter the reaction products formed on the enamel surface and even the total amount of fluoride adsorbed on the surface. No study has examined the role of the organic layer present on the surface of newly erupted enamel (Nasmyth's membrane; Primary enamel cuticle) as it affects fluoride uptake and the reaction products formed following topical application to newly erupted teeth. This study was designed to observe the fluoride uptake and surface reaction products formed following removal of the surface organic layer by different means.

Hypothesis

There is a difference in the fluoride uptake and reaction products formed in the presence or absence of the surface organic layer in fully formed pre-eruptively mature unerupted teeth after topical fluoride application.
Specific Objectives

1. To study the effects of toothbrushing/deproteinizing treatments on topical fluoride uptake in fully formed pre-eruptively mature enamel.

2. To determine the type of fluoride deposited following topical fluoride exposure.

3. Measure fluoride levels at different enamel depths after treatment with different concentrations of topical fluoride solutions.

4. To study the surface reaction products after the fluoride treatment by scanning electron microscopy.
Materials and Methods

Enamel samples: Human unerupted third molars with more than half their roots formed were used to ensure that the specimens of enamel were mature (Fejerskov et al, 1984; Nyvad et al, 1988).

Storage of teeth: The surgically removed unerupted third molars were stored at 4°C on cotton soaked with distilled water containing thymol crystals (Nyvad et al, 1988). Soft tissue tags, if any, were cut at the cervix with scissors to prevent physical interference with the treatments. The teeth were analyzed within 21 days of storage to avoid any possible modification in the surface organic layer following prolonged storage.

Number of teeth: The first block consisted of 90 teeth and after treatment, the teeth were chemically analyzed for fluoride uptake and reaction products. The second block consisted of 18 teeth and after treatment, the teeth were morphologically analyzed for surface reaction products by scanning electron microscopy.

Experimental groups: There were nine experimental groups:
I. No toothbrushing /deproteinizing and no fluoride treatment
II. No toothbrushing /deproteinizing and 2% NaF (9048 ppm F⁻) treatment
III. No toothbrushing /deproteinizing and 1 ppm Fluoride treatment
IV. Toothbrushing and no fluoride treatment
V. Toothbrushing and 2% NaF (9048 ppm F⁻) treatment
VI. Toothbrushing and 1 ppm Fluoride treatment
VII. Deproteinizing and no fluoride treatment
VIII. Deproteinizing and 2% NaF (9048 ppm F⁻) treatment

IX. Deproteinizing and 1 ppm Fluoride treatment

**Allocation of teeth:** Balanced block randomization (Petrie, 1982) was used to allocate the teeth to the various treatment groups. The teeth were randomly allocated to the nine experimental groups according to a random sequence obtained from a table of random numbers (Petrie, 1978) (Appendices 1 and 2).

**Treatment of specimens:** Following removal from the storage solution, each tooth was rinsed in a stream of 50 ml of deionized water. Further treatment of the tooth was based upon the individual group allocation.

**Toothbrushing treatment:** Each surface of the tooth received toothbrushing (20 strokes) with deionized water using a soft (Oral-B Ultra P-20) toothbrush. After toothbrushing, the teeth were rinsed in a stream of 50 ml of deionized water.

**Deproteinizing treatment:** The tooth was immersed in a plastic bottle containing 100 ml of 0.1M Urea solution for seven days at 37°C (Arends et al, 1984). The solution was not stirred. Upon removal from the Urea solution, the teeth were rinsed in a stream of 50 ml of deionized water.

**Topical fluoride treatment:** The tooth received a four minute treatment with 10 ml of aqueous non-buffered 2% NaF (9048 ppm F⁻) solution in a glass beaker or a 24 hour treatment with 10 ml of aqueous NaF (1 ppm F⁻) solution in a plastic bottle. Following the fluoride treatment, the teeth were rinsed in a stream of 500 ml of deionized water to remove unreacted fluoride similar to
rinsing in running tap water for 30 seconds (Dijkman and Arends, 1988) or rinsing in running distilled water for 10 seconds (Sieck et al, 1990).

**Preparation of specimens:** Specimens for fluoride assay were prepared as follows. The teeth were dried in air and the roots were cut off followed by sectioning of the crowns into two equal specimens. Then an rectangular waterproof adhesive tape with a window (10.65 mm²) was applied to each specimen. The window on the enamel surface of each specimen provided an standardized area of enamel biopsy (Aasenden et al, 1972); the rest of the specimen was covered with yellow sticky wax (Wefel and Harless, 1981). Following removal from KOH solution, nail varnish was painted over the sticky wax as the sticky wax became brittle following exposure to KOH solution. For scanning electron microscopy, the teeth were dried in air and the roots were cut off followed by sectioning of the crowns into two approximately equal specimens each. Care was taken to minimize the handling of the crown surfaces to avoid disturbing the surface morphology.

**Fluoride assay:** The specimen was washed in 1M KOH solution (10 ml) for 24 hours (Caslavska et al, 1975) to remove the soluble fluoride (calcium fluoride) as Caslavska et al (1975) observed that calcium fluoride (alkali-soluble fluoride) on enamel is readily removed in vitro by KOH in contrast to fluorapatite which is insoluble in KOH. The alkali method has limitations because calcium fluoride that has been exposed to phosphate will be underestimated (Saxegaard, 1988). However, trace amounts of phosphate, possibly present during the alkali treatment would probably not interfere with the dissolution rate of calcium fluoride in alkali, because the presence of HPO₄²⁻ ions, which are essential for dissolution reduction, would be
negligible at this high pH (Saxegaard, 1988). The specimen was immersed in 1M KOH solution in a plastic bottle for 24 hours (Dijkman et al, 1982). The solution was not stirred. After removal from the KOH solution, the specimens were rinsed with 200 ml of deionized water. The alkaline KOH solution was brought to a pH of 5.3 - 5.6 by the addition of 1M HNO₃ (Dijkman et al, 1982) and low-level Tisab and then analyzed for alkali-soluble fluoride.

Following the KOH wash, the specimens were further assayed for bound fluoride by acid etching. The specimens received an acid-etch biopsy (Cooley, 1961) for a standardized period of time (Muhlemann et al, 1964). The specimens received 15-, 30- and 60- second exposures of 0.5M Perchloric acid (0.5 ml) followed by a rinse in low-level Tisab (0.5 ml) (Clarkson et al, 1988). The resultant solutions were analyzed for fluoride with a fluoride ion electrode (McCann, 1968). Fluoride was determined with a combination fluoride electrode (Orion Model 96-09), coupled with an ionalyzer (Orion Model 901). From the fluoride ion concentration, the amount of fluoride obtained in each enamel layer was determined (Brudevold et al, 1968).

The method to calculate fluoride concentrations at different depths was as follows. The weight of enamel removed in one etch period was too small to be determined directly. Therefore, the amount of enamel in the etch solution was calculated based on the phosphorous content (Muhlemann et al, 1964). The total quantity of enamel in the etch solution was calculated assuming an phosphorous content in enamel of 18.1% (Angmar et al, 1963) and enamel density of 2.95 gm/cm³ (Manly et al, 1939). Phosphorous concentration was determined spectrophotometrically after the method of
Chen et al (1956) using wet phosphate standards. The total amount of fluoride in the etch solution was calculated based on the enamel weight and fluoride ion concentration (Brudevold et al, 1968). The depth of single biopsies was calculated from the enamel weight and the biopsy area and the density of enamel (Bruun, 1973) (Appendix 3).

**Scanning electron microscopy:** Scanning electron microscope was used to study the surface topography of enamel (Hoffman et al, 1969) and to note the surface reaction products of fluoride treatments. SEM cannot be used to distinguish the difference between hydroxyapatite and fluorapatite due to the similarity in crystalline appearance and the small crystalline size, however it can be used to differentiate spherical appearing granular material suggestive of calcium fluoride (Wei, 1975).

One specimen from each tooth was immersed in 1M KOH solution in a plastic bottle for 24 hours; the solution was not stirred. After removal from the KOH solution, the specimens were rinsed with 200 ml of deionized water. The other specimen from the same tooth did not receive this KOH treatment.

The specimens for SEM were air-dried and mounted on aluminum SEM stubs with colloidal graphite and then placed in a dessicator. Once sufficiently dehydrated, the sections were coated using a gold source and observed in a scanning electron microscope at 15-25 kV for surface reaction products. The magnifications used were X 1000 and X 5000. Each specimen was scanned from left to right (four views). The middle third of the buccal/lingual surface of each specimen was scanned.
**Statistical analysis:** According to the Central Limit Theorem, simple random samples drawn from an infinite population that has a mean and a standard deviation, then the distribution of means always tends toward normality as the sample size increases (Bowen and Starr, 1982). Mean group values of the following parameters were obtained:

- Mean amount of alkali-soluble fluoride (µg)
- Mean fluoride (ppm) in 15 second etch
- Mean biopsy depth (µm) in 15 second etch
- Mean fluoride (ppm) in 30 second etch
- Mean biopsy depth (µm) in 30 second etch
- Mean fluoride (ppm) in 60 second etch
- Mean biopsy depth (µm) in 60 second etch

All mean values were calculated with ± one standard deviation.

Group analyses included coefficients of variance and ranges. Comparative analysis (ANOVA) was done to determine statistical differences between the groups. Also, within each group, one specimen from each tooth was allocated to one of two sub-groups, followed by comparison of the two sub-groups using ANOVA (Group Intra-tooth analysis). Level of statistical significance for the ANOVA was set at p=0.05 (95%).
Limitations of the Experiment

1. Teeth unexposed to the oral environment were used to avoid the acquired pellicle formed on erupted teeth. Hence, the organic layer on the enamel surface was essentially developmental in nature without exposure to the oral environment and possible structural modifications.

2. Some of the organic material might have been dental follicle.

3. Pre-eruptive enamel maturation has been correlated to root development.

4. The organic layer might have been damaged/removed during the surgical extraction/specimen preparation.

5. The organic layer might have altered during storage.
Results

A pilot study demonstrated the presence of a surface organic layer of variable extent on the enamel surface of fully formed pre-eruptively mature third molars (Appendix 4). It was also observed that this layer was removed by toothbrushing (Fig. 1) as well as deproteinizing (Fig. 2) surface treatments.

i) Chemical assay

Wide variation in sample measurements within each group was noted with high standard deviations (Tables 2 and 3), coefficients of variance (Table 4) and ranges (Table 5). Bound fluoride levels in all the groups decreased with increasing etch depth (Table 2). Etch depths in all the groups increased with increased duration of exposure to 0.5M Perchloric acid (Table 3).

Alkali-soluble fluoride analysis*: Alkali-soluble fluoride analysis for the three groups in which the surface organic layer was not removed (Groups I, II and III) showed a significantly greater fluoride level in the 2% NaF (9048 ppm F⁻) group (p=0.001) as compared to the 1 ppm fluoride and control groups. The 1 ppm fluoride group did not differ from the control group (Table 6).

Alkali-soluble fluoride analysis for the three groups in which the surface organic layer was removed by toothbrushing (Groups IV, V and VI) showed a significantly greater fluoride level in the 2% NaF (9048 ppm F⁻) group (p=0.001) as compared to the 1 ppm fluoride and control groups. The 1 ppm fluoride group did not differ from the control group (Table 6).
Alkali-soluble fluoride analysis for the three groups in which the surface organic layer was removed by deproteinizing (Groups VII, VIII and IX) showed a significantly greater fluoride level in the 2% NaF (9048 ppm F⁻) group as compared to the 1 ppm fluoride (p=0.05) and control (p=0.01) groups. The 1 ppm fluoride group did not differ from the control group (Table 6).

Alkali-soluble fluoride analysis for the three groups which did not receive any fluoride treatment (Groups I, IV and VII) showed no significant difference (p=0.05) in fluoride levels following removal of the surface organic layer by toothbrushing/deproteinizing treatments (Table 6).

Alkali-soluble fluoride analysis for the three 2% NaF (9048 ppm F⁻) treatment groups (Groups II, V and VIII) showed no significant difference (p=0.05) in fluoride levels following removal of the surface organic layer by toothbrushing/deproteinizing treatments (Table 6).

Alkali-soluble fluoride analysis for the three 1 ppm fluoride treatment groups (Groups III, VI and IX) showed no significant difference (p=0.05) in fluoride levels following removal of the surface organic layer by toothbrushing/deproteinizing treatments (Table 6).

* [Absolute alkali-soluble fluoride values have to be interpreted with caution and may be high because of technique error (Appendix 5)].

**Bound fluoride analysis:** Comparison of 15 sec-, 30 sec- and 60 sec- etch bound fluoride levels between the various groups showed no significant differences (p=0.05) following removal of the surface organic layer by toothbrushing
Deproteinizing treatment significantly reduced (p=0.05) bound fluoride levels in Group VII as compared to no surface treatment in Group I in the 15 sec- and 30 sec- etches (Tables 7 and 8). Comparison of the three deproteinizing groups (Groups VII, VIII and IX) showed significantly greater bound fluoride level in the 2% NaF (9048 ppm F⁻) treatment group as compared to the 1 ppm fluoride treatment (p=0.05) and control (p=0.001) groups in the 15 sec- etch (Table 7). This difference was also noted in the 30 sec- etch with the 2% NaF (9048 ppm F⁻) treatment group having a significantly greater bound fluoride level as compared to the 1 ppm fluoride treatment (p=0.01) and control (p=0.01) groups (Table 8). However, in the 60 sec- etch, the 2% NaF (9048 ppm F⁻) treatment group showed a significantly greater bound fluoride level than only 1 ppm fluoride treatment (p=0.05) group (Table 9).

Other differences noted are likely to be alpha error because no definitive trend was observed.

Etch depth analysis: Comparison of 15 sec-, 30 sec- and 60 sec- etch depths between the various groups showed no significant differences following the various surface/fluoride treatments (Tables 10, 11 and 12). The only difference noted (15 sec- etch: Group VI vs. Group IX) is likely to be alpha error because of the small magnitude of difference.
**Group Intra-tooth analysis:** Comparison of the two sub-groups (consisting of one specimen from each tooth) showed no significant difference (p=0.05) in any of the groups for the parameters studied (Table 13).

ii) **Morphologic assay**

Large variability was noted in different views of the same specimen in scanning electron microscopy. Typical morphologic appearance of each specimen was considered for description and comparison.

Enamel surface of the teeth without any surface/fluoride treatment (Group I) showed sparse irregularly shaped deposits; partially occluded pores and focal holes (Fig. 3a). KOH treatment of these surfaces resulted in the removal of most of the sparse irregularly shaped deposits on the surface and also of the material occluding the pores (Fig. 3b). Neither toothbrushing treatment (Group IV) (Fig. 4) nor deproteinizing treatment (Group VII) (Fig. 5) modified the enamel surface.

Teeth with 2% NaF (9048 ppm F⁻) treatment of the enamel (without any surface treatment) (Group II) showed numerous irregularly shaped surface deposits (Fig. 6a). Higher magnification (X 5000) of these deposits only showed large irregular deposits (Fig. 6b). Most of these deposits were removed by KOH treatment (Fig. 6c). Toothbrushing treatment (Group V) (Fig. 7) or deproteinizing treatment (Group VIII) (Fig. 8) did not modify the surface interaction (morphologic) between the 2% NaF (9048 ppm F⁻) solution and the enamel.
Teeth subjected to 1 ppm fluoride treatment of the enamel (without any surface treatment) (Group III) showed sparse irregularly shaped deposits with the morphologic appearance not different from that of the teeth without any fluoride treatment (Fig. 9a). KOH treatment of these surfaces also removed the sparse deposits as on teeth without any fluoride treatment (Fig. 9b). Toothbrushing treatment (Group VI) (Fig. 10) or deproteinizing treatment (Group IX) (Fig. 11) did not modify the surface interaction (morphologic) between the 1 ppm fluoride solution and the enamel.
Discussion

Newly erupted teeth are more susceptible to caries and this susceptibility decreases with increased exposure to the oral cavity (Carlos and Gittelsohn, 1965; King et al, 1980). Topical fluorides have been shown to be more effective in controlling caries in teeth that erupt during a program of topical fluoride exposure than those already present at baseline (Muhler, 1960; Averill et al, 1967; Horowitz and Heifetz, 1969; DePaola and Mellberg, 1973). This study examined the role of the surface organic layer in the interaction between newly erupted enamel (pre-eruptively mature enamel in unerupted teeth) and topical fluorides. In a pilot study the presence of a surface organic layer of variable extent was demonstrated on the enamel surface of fully formed pre-eruptively mature third molars. It was also observed that this layer was removed by toothbrushing as well as deproteinizing surface treatments.

Significant fluoride uptake was observed only in the 2% NaF (9048 ppm F⁻) treatment groups as compared with the 1 ppm fluoride treatment groups, regardless of the surface treatment on enamel. Alkali-soluble fluoride was the primary surface reaction product formed following 2% NaF (9048 ppm F⁻) application. This is in accordance with earlier observations that calcium fluoride (alkali-soluble fluoride) is the major product deposited on enamel after exposure to high concentrations of fluoride (Baud and Bang, 1970; Caslavska et al, 1975; Saxegaard and Rolla, 1988). It has been observed that increased time of exposure, lowered pH of fluoride agent, and increased fluoride concentration were effective means of increasing KOH-soluble and KOH-insoluble fluoride (Saxegaard and Rolla, 1988). The results of this study
confirm that increased fluoride concentration (2% NaF) increases KOH-soluble (alkali-soluble) fluoride. However, a similar increase in KOH-insoluble (bound fluoride) levels were not seen. The reason for this lack of increase in bound fluoride levels might be due to the pH of the fluoride agent. This may be due to treatment with fluoride agent at neutral pH because as Saxegaard and Rolla (1988) observed significant uptake of bound fluoride only following topical fluoride application at a low pH. Perhaps significant fluoride uptake (alkali-soluble and alkali-insoluble) might have occurred in the 1 ppm fluoride treatment groups if the exposure time had been longer than 24 hours as Saxegaard and Rolla (1988) noted that increased time of exposure increases both KOH-soluble and KOH-insoluble fluoride levels.

Alkali-soluble fluoride was detected in all the groups leading to the observation that this fluoride is present on all teeth. However, the fluoride levels following alkali treatment in teeth other than those exposed to 2% NaF (9048 ppm F⁻) solution were low. This amount is at the detection limit of the fluoride electrode and therefore may have no significance as reported earlier by Saxegaard and Rolla (1988). Alkali-soluble fluoride levels for the groups which did not receive any fluoride treatment or received 1 ppm fluoride treatment showed no differences (fluoride levels detected were at the detection limit of the fluoride electrode). Comparison of the three 2% NaF (9048 ppm F⁻) groups showed no significant difference in the alkali-soluble fluoride levels regardless of surface treatment. Although there were no statistical differences in fluoride levels between the groups, there is still a need to explain the greater cariostatic effect of topical fluorides on newly erupted teeth. A pilot study did demonstrate removal of the organic material
on the surface by toothbrushing and also by deproteinizing. Probably the organic layer affects the quality of the reaction product.

Bound fluoride levels in all the groups decreased with increasing etch depth confirming previous observations (Speirs, 1971; Weatherell et al, 1973). Bound fluoride (KOH-insoluble) levels between the various groups showed no differences following removal of the surface layer by toothbrushing /deproteinizing surface treatments or following 2% NaF (9048 ppm F⁻) / 1 ppm fluoride treatment. The only exception was that deproteinizing surface treatment reduced bound fluoride levels. However, this reduced bound fluoride level following deproteinizing was reversed and normal levels achieved following 2% NaF (9048 ppm F⁻) application. Arends et al (1984) observed that 0.1M Urea removes organic material normally plugging natural defects in the enamel surface. It is likely that removal of this material possibly with associated fluoride reduced bound fluoride levels; and that 2% NaF (9048 ppm F⁻) treatment restored the bound fluoride levels because of unimpeded access into the enamel. This aspect will be discussed further in this section.

Etch depths in all the groups increased with increased duration of exposure to 0.5M Perchloric acid confirming previous observations that the average thickness of the enamel layer removed is a function of the total etching time (Arends and Schuthof, 1975). Comparison of the etch depths between the various groups showed no significant differences following the various surface/fluoride treatments. Therefore acid solubility (to 0.5M Perchloric acid) is not modified by toothbrushing/deproteinizing surface treatments and/or 2% NaF (9048 ppm F⁻) /1ppm fluoride treatments.
The organic material in the surface in fully formed pre-eruptively mature enamel other than the primary enamel cuticle are the enamel matrix proteins, amelogenins and enamelin. Termine et al (1980) observed that in mature enamel matrix, the proportion of enamelin is more than amelogenins. The enamelin are associated with the mineral phase of the enamel (Termine et al, 1979). Therefore the organic material removed by the surface treatments in this study (particularly deproteinizing) can only be amelogenins in addition to the primary enamel cuticle. Since the amelogenins are the non-structural, hetero-dispersed particulate material in the inter-crystallite space (B'ai and Warshawsky, 1985), the resulting porosity (following deproteinizing) should have improved the access of fluoride into the enamel; however, this porosity did not improve alkali-soluble fluoride uptake. Increased alkali-soluble fluoride uptake might have been possible if the fluoride had formed at least a loose bond in the liquid phase (within enamel).

It was observed that deproteinizing reduced bound fluoride levels; however, this reduced bound fluoride level following deproteinizing was reversed and normal levels achieved following 2% NaF (9048 ppm F⁻) application. This was the only bound fluoride uptake noted in this study following 2% NaF (9048 ppm F⁻) application. It is suggested that during deproteinizing, the bound fluoride possibly associated with the amelogenins is removed. Following 2% NaF (9048 ppm F⁻) application, this bound fluoride level is restored possibly in association with the enamelin or the enamel crystallites.
Robinson et al (1982) noted that there is a tendency for smaller molecular weight material to persist in the maturing enamel after most of the matrix had been withdrawn. Aoba et al (1987) noted that parent amelogenins adsorb onto surface of hydroxyapatite crystals in contrast to the smaller polypeptides derived from degradation of the intact molecule; therefore these moieties are likely to be present as free entities in the liquid phase within enamel. Chander et al (1982) showed that the calcium fluoride crystal changes the charge from positive to negative on exposure to phosphate ions and this increased the protein-binding capacity of the calcium fluoride. Rykke et al (1989) in an in vitro study demonstrated that calcium fluoride took up small amounts of proteins as compared with hydroxyapatite; however, when the calcium fluoride was pre-treated with a phosphate buffer, pH 6.8, the protein adsorption increased markedly. Therefore it is likely that the amelogenins (present along with significant amounts of phosphate in pre-eruptively mature enamel) bind fluoride (alkali-soluble fluoride) and may maintain high fluoride concentrations in the liquid phase within enamel. This will promote crystal growth processes as Arends and Christoffersen (1990) noted that crystal growth processes may require high values of fluoride in the liquid phase (within enamel) in order to form fluorapatite and hydroxyapatite.

Lussi et al (1988) noted no in vitro binding of fluoride to isolated organic matrix of secretory bovine enamel, but the properties of the organic matrix differs between the secretory and mature stages. In the secretory stage, the principal matrix proteins are the hydrophobic amelogenins (Fincham, 1982) which adsorb onto hydroxyapatite crystals with resultant retardation of fluoride incorporation (Aoba et al, 1989). However in the mature stage, the principal matrix proteins are the enamelins (Termine et al, 1980) which
regulate the continuous growth in width and thickness of enamel crystallites during maturation (Bai and Warshawsky, 1985). The enamelins are associated with the mineral phase of the enamel (Termine et al, 1979). They might persist in the maturing tissue after most of the matrix had been withdrawn as Robinson et al (1982) observed a 55 kD material in the maturing tissue after withdrawal of organic matrix; this material increased dramatically when the enamel was treated with a demineralizing agent. They also observed that when enamel was incubated at 37°C in a moist environment, this material was released from mineral; this release was not inhibited by proteolytic inhibitors but was reduced by fluoride ion. This suggests interaction between matrix proteins in the mature enamel and fluoride.

Crabb (1976) noted that human teeth erupt with an incompletely mature enamel and porous outer layer of surface enamel. Therefore, this porosity is likely to have improved fluoride uptake as observed by Deutsch and Shapira (1987). Fearnhead et al (1982) stated that even in the presence of an adequate ion population (fluoride) and accessible diffusion pathways, the presence of an organic "contaminant" might retard or arrest further growth of the apatite crystals already present. This might be true in old enamel as Robinson et al (1990) demonstrated improved ion (45Ca2+) uptake by removal (deproteinizing) of organic material in natural carious lesions in vitro. However, this is contrary to our observations where removal of the organic material (particularly by deproteinizing) did not significantly affect alkali-soluble fluoride uptake; although it is expected that the removal of the organic material might have increased the porosity and opened the diffusion pathways. However, there was some bound fluoride uptake following 2% NaF (9048 ppm F−) application which reversed (to normal levels) reduced
bound fluoride levels following deproteinizing. Therefore, it appears that improved porosity of newly erupted porous enamel may not be a major factor in fluoride uptake.

The organic material (removed by toothbrushing and possibly primary enamel cuticle) on the enamel surface might act as a semi-permeable membrane and may promote fluoride ion uptake into the liquid phase (within enamel) because of the Donnan membrane effect. Donnan membrane effect is likely to be established because the calcium fluoride crystals (assuming the alkali-soluble fluoride is indeed calcium fluoride) changes the charge from positive to negative on exposure to phosphate ions (Chander et al, 1982) in pre-eruptively mature enamel. However, the experimental evidence from this study does not corroborate this Donnan membrane hypothesis.

It has been suggested that this alkali-soluble fluoride is calcium fluoride or calcium fluoride-like material (Caslavska et al, 1975). The chemical composition and solubility product of “calcium fluoride-like material” formed in vivo are not known; the only information available is that the material is “calcium fluoride-like” and the solubility is very low (Arends and Christoffersen, 1990). Dijkman and Arends (1988) noted that after one week in situ, the calcium fluoride-like material is mostly lost either through “chipping away” from the surface or as local dissolution with subsequent leaching of Ca^{2+} and F through the pellicle. Saxegaard and Rolla (1989) showed in vivo that after single topical fluoride application to sound enamel, the loss of calcium fluoride (alkali-soluble fluoride) was arrested after 1-2 days at 70% of the original level. They showed that a slight increase in the
amount of firmly bound fluoride was significant after two days, possibly due to transformation of calcium fluoride on enamel. Chander et al (1982) showed that calcium fluoride transforms to fluorapatite on exposure to phosphate ions. It has been speculated that calcium fluoride deposited on enamel forms a dissolution-limiting phase along with the phosphate ions in saliva (Saxegaard et al, 1988).

Intra-oral caries model study noted that only KOH-soluble fluoride reduced mineral loss and lesion depths significantly (Ogaard et al, 1990). It is generally agreed that the major effects of fluoride ions towards enhancing crystal growth and retarding dissolution of enamel are derived from the contribution of fluoride ion in the solution phase to increased supersaturation or decreased undersaturation (White and Nancollas, 1990). Loosely bound fluoride has been suggested to act as a potential "reservoir" or "depot" source of solution fluoride (White and Nancollas, 1990) that releases significant amounts of fluoride ions from calcium fluoride in both neutral and acid solutions whereas firmly bound fluoride (fluorapatite and fluorhydroxyapatite) releases fluoride ions only in acid solutions (White and Nancollas, 1990).

The amount of fluoride uptake following topical fluoride exposure may not be the only critical factor in cariostasis as Ogaard et al (1988) showed that shark enamel consisting of nearly pure fluorapatite (30,000 ppm fluoride) develops caries. Most likely there is a dynamic equilibrium between fluoride in solution (within enamel) and adsorbed fluoride (on enamel crystallites) at the crystal surface interface; when the crystallite is completely covered by adsorbed fluoride, there is a maximum inhibition of dissolution (Arends and
Christoffersen, 1990). If, due to a high fluoride concentration in the liquid phase (within enamel), adsorbed fluoride (on enamel crystallites) is at its maximum value, further increase of fluoride concentration in the liquid phase (within enamel) leads to crystal growth to form fluorapatite and hydroxyapatite (Arends and Christoffersen, 1990). In vivo formed "calcium fluoride-like material" can contribute to adsorbed fluoride (on enamel crystallites); however, the value of fluoride in the liquid phase (within enamel) and therefore the adsorbed fluoride (on enamel crystallites) resulting from this loosely bound fluoride is not known (Arends and Christoffersen, 1990).

The enamel surfaces of the unerupted third molars (with mature enamel) showed sparse irregularly shaped deposits, partially occluded pores and focal holes. This is in agreement with the earlier observations of Nyvad et al. (1988) who noted that scanning electron microscopy of unerupted third molars (with mature enamel) demonstrated numerous irregular holes (focal holes) and flat enamel caps, particularly in the middle third of the enamel surfaces; the bottom of the holes were partly obscured by an amorphous substance.

1M KOH treatment (24 hours) of the enamel surfaces resulted in the removal of most of the sparse irregularly shaped deposits on the surface and also of the material occluding the pores. This suggests that some of the material on the mature enamel of unerupted third molars is alkali-soluble. Toothbrushing and deproteinizing surface treatments did not modify the appearance of the enamel surface.
This is contrary to the earlier report of Arends et al (1984) who observed that 0.1M Urea does not attack the organic matrix between the crystallites but removes the organic material normally plugging natural defects in the enamel surfaces, especially in the perikymata. They noted that the typical features seen on enamel surface after exposure to 0.1M Urea solution (for seven days at 37°C) are local attack in perikymata as well as local “pitting” (hole formation). \( {^{14}} \text{C}\)-labeled Urea (1 mmol) in one week penetrates more than 50 μm depth into enamel at 37°C with a diffusion constant (of Urea in enamel) in the order of \( 10^{-10} \text{cm}^2.\text{s}^{-1} \) (Arends et al, 1984).

Teeth with 2% NaF (9048 ppm F⁻) treatment of the enamel showed numerous irregularly shaped deposits. This is contrary to earlier reports that globular deposits are seen following topical fluoride exposure (Barbakow et al, 1983, 1984; Saxegaard et al, 1988). However the globular deposits on sound enamel have been reported only at pH values 6 and below (Barbakow et al, 1983) or time of exposure longer than one hour at neutral pH (Saxegaard and Rolla, 1988). Since the pH of the 2% NaF (9048 ppm F⁻) solution in this experiment was greater than 6 and also the time of exposure was only four minutes, the results are not a contradiction. However, these results emphasize the need to simulate clinical treatment conditions closely to obtain realistic morphologic assessments. Most of the deposits formed following 2% NaF (9048 ppm F⁻) treatment were removed by 1M KOH (24 hours) treatment demonstrating that this surface reaction product is alkali-soluble. This is in agreement with the earlier findings of Clarkson et al (1988) who demonstrated no surface deposits on APF treated (four minutes) sound enamel following a 24 hour wash in 1M KOH. Toothbrushing or deproteinizing surface treatments did not modify the surface interaction
(morphologic) between the 2% NaF (9048 ppm F⁻) solution and the enamel. This demonstrates that the surface organic layer on unerupted teeth plays no role in the deposition of surface reaction products on enamel. This is similar to the reported incapacity of the acquired pellicle to modify the deposition of alkali-soluble fluoride on enamel in vitro following a single topical application of 2% NaF solution at neutral pH (Cruz and Rolla, 1991).

Teeth with 1 ppm fluoride treatment of the enamel showed sparse irregularly shaped deposits with morphologic appearance not different from that of teeth without any fluoride treatment. Toothbrushing or deproteinizing surface treatments did not modify the surface interaction (morphologic) between the 1 ppm fluoride solution and the enamel.

It is speculated (from literature review) that the greater cariostatic effect of topical fluorides on newly erupted enamel may be due to the following reasons:

a) the porous enamel allows diffusion of fluoride into the liquid phase within enamel

b) alkali-soluble fluoride deposited on the enamel surface following topical fluoride application may act as a “reservoir” of fluoride ions to increase fluoride concentration in the liquid phase within enamel

c) amelogenins (enamel matrix proteins) may bind fluoride to maintain increased fluoride concentration in the liquid phase within enamel

d) increased fluoride concentration in the liquid phase (within enamel) improves the enamel crystallite properties

e) the fluoride uptake modified by the surface organic layer in newly erupted enamel is quantitatively insignificant but might qualitatively
improve the uptake leading to the reported greater cariostatic effect of topical fluorides on newly erupted teeth.
Conclusions

- Significant fluoride uptake in pre-eruptively mature enamel occurred only following 2% NaF (9048 ppm F⁻) application as compared with 1 ppm fluoride treatment.
- Alkali-soluble fluoride was the primary surface reaction product formed following 2% NaF (9048 ppm F⁻) application in pre-eruptively mature enamel.
- Removal of the surface organic layer by toothbrushing/deproteinizing did not significantly affect fluoride uptake in pre-eruptively mature enamel.
- Deproteinizing surface treatment reduced bound fluoride levels in pre-eruptively mature enamel. However, this reduced bound fluoride level following deproteinizing was reversed and normal levels achieved following 2% NaF (9048 ppm F⁻) application.
- Acid solubility (to 0.5M Perchloric acid) in pre-eruptively mature enamel was not modified by toothbrushing/deproteinizing surface treatments and/or 2% NaF (9048 ppm F⁻)/1ppm fluoride applications.
- Toothbrushing and deproteinizing treatments did not modify (seen in SEM) the surface morphology in pre-eruptively mature enamel.
- Numerous irregularly shaped surface deposits (seen in SEM) were noted in pre-eruptively mature enamel that was treated with 2% NaF (9048 ppm F⁻) application. These deposits were alkali-soluble.
- Toothbrushing/deproteinizing surface treatments did not modify the interaction (morphologic) between the pre-eruptively mature enamel surface and fluoride solutions.
Summary

Newly erupted teeth are more susceptible to caries and this susceptibility decreases with increased exposure to the oral cavity. However, this caries susceptibility of newly erupted teeth is reduced by topical fluoride exposure. This in vitro study examined the role of the surface organic layer in new enamel (fully formed pre-eruptively mature enamel in unerupted teeth) on fluoride uptake following topical fluoride exposure. A pilot study demonstrated the presence of a surface organic layer on the enamel of fully formed pre-eruptively mature third molars; this layer was removed by toothbrushing as well as deproteinizing surface treatments.

The surface organic layer was removed by either toothbrushing or deproteinizing (in 0.1M Urea for seven days at 37°C). The topical fluoride exposure included either 2% NaF (9048 ppm F\textsuperscript{-}) solution for four minutes or 1 ppm fluoride solution for 24 hours. Following fluoride treatment, alkali-soluble fluoride was extracted with 1M KOH solution for 24 hours followed by acid etch analysis (0.5M HClO\textsubscript{4}) for bound fluoride.

The results showed that significant fluoride uptake occurred only following the 2% NaF (9048 ppm F\textsuperscript{-}) application as compared to the 1 ppm fluoride application. Alkali-soluble fluoride was the primary surface reaction product formed following 2% NaF (9048 ppm F\textsuperscript{-}) application. Removal of the surface organic layer by toothbrushing /deproteinizing did not affect fluoride uptake. The only exception being that deproteinizing reduced bound fluoride levels; however, this reduced bound fluoride level following deproteinizing was reversed and normal levels achieved following 2% NaF
(9048 ppm F⁻) application. Acid solubility (to 0.5M Perchloric acid) was not modified by toothbrushing /deproteinizing surface treatments and/or 2% NaF (9048 ppm F⁻)/1ppm fluoride applications.

Toothbrushing and deproteinizing treatments did not modify (seen in SEM) the surface morphology in pre-eruptively mature enamel. Scanning electron microscopy demonstrated numerous irregularly shaped surface deposits in teeth that were treated with 2% NaF (9048 ppm F⁻) application. These deposits were alkali-soluble. Toothbrushing /deproteinizing surface treatments did not modify the interaction (morphologic) between the pre-eruptively mature enamel surface and fluoride solutions.

It is speculated (from literature review) that the greater cariostatic effect of topical fluorides in newly erupted teeth may be due to the following reasons:

a) the porous enamel allows diffusion of fluoride into the liquid phase within enamel

b) alkali-soluble fluoride deposited on the enamel surface may act as a "reservoir" of fluoride ions to increase fluoride concentration in the liquid phase within enamel

c) amelogenins (enamel matrix proteins) may bind fluoride to maintain increased fluoride concentration in the liquid phase within enamel

d) increased fluoride concentration in the liquid phase (within enamel) improves the enamel crystallite properties

e) the fluoride uptake modified by the surface organic layer in newly erupted enamel is quantitatively insignificant but might qualitatively
improve the uptake leading to the reported greater cariostatic effect of topical fluorides on newly erupted teeth.
<table>
<thead>
<tr>
<th>Surface Treatment</th>
<th>No Surface Treatment</th>
<th>Toothbrushing</th>
<th>Deproteinizing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluoride Treatment</td>
<td>No Fluoride Treatment</td>
<td>Group I</td>
<td>Group II</td>
</tr>
<tr>
<td></td>
<td>2% Sodium Fluoride Treatment</td>
<td>Group III</td>
<td>Group IV</td>
</tr>
<tr>
<td></td>
<td>1 ppm Fluoride Treatment</td>
<td>Group VI</td>
<td>Group V</td>
</tr>
<tr>
<td></td>
<td>2 ppm Fluoride Treatment</td>
<td>Group VII</td>
<td>Group VIII</td>
</tr>
<tr>
<td></td>
<td>3 ppm Fluoride Treatment</td>
<td>Group IX</td>
<td>Group IX</td>
</tr>
</tbody>
</table>

Table 1
Fluoride Concentration Means and Standard Deviations

<table>
<thead>
<tr>
<th>Fluoride</th>
<th>No Surface treatment</th>
<th>Toothbrushing</th>
<th>Deproteinizing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No F Group I</td>
<td>2% NaF Group II</td>
<td>1 ppm F Group III</td>
</tr>
<tr>
<td>Alkali-Soluble fluoride (µg)*</td>
<td>0.61 ± 1.01</td>
<td>1.69 ± 1.17</td>
<td>0.49 ± 0.44</td>
</tr>
</tbody>
</table>

Bound fluoride (ppm):

<table>
<thead>
<tr>
<th></th>
<th>15 sec etch fluoride</th>
<th>30 sec etch fluoride</th>
<th>60 sec etch fluoride</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2010.19 ± 936.62</td>
<td>1570.45 ± 659.71</td>
<td>1144.77 ± 544.94</td>
</tr>
<tr>
<td></td>
<td>2333.44 ± 773.95</td>
<td>1877.14 ± 921.75</td>
<td>1327.42 ± 682.32</td>
</tr>
<tr>
<td></td>
<td>1817.17 ± 756.40</td>
<td>1228.30 ± 440.46</td>
<td>873.29 ± 303.44</td>
</tr>
<tr>
<td></td>
<td>1735.82 ± 663.67</td>
<td>1531.99 ± 831.66</td>
<td>1193.38 ± 1245.07</td>
</tr>
<tr>
<td></td>
<td>1944.87 ± 705.49</td>
<td>1536.03 ± 1203.04</td>
<td>987.67 ± 290.34</td>
</tr>
<tr>
<td></td>
<td>12129.81 ± 1195.71</td>
<td>1448.80 ± 419.52</td>
<td>1245.61 ± 645.00</td>
</tr>
<tr>
<td></td>
<td>1571.79 ± 638.18</td>
<td>1234.21 ± 308.98</td>
<td>970.84 ± 320.87</td>
</tr>
<tr>
<td></td>
<td>2317.48 ± 798.51</td>
<td>1689.68 ± 639.56</td>
<td>1165.69 ± 490.63</td>
</tr>
<tr>
<td></td>
<td>1868.83 ± 835.33</td>
<td>1265.38 ± 474.43</td>
<td>901.49 ± 274.47</td>
</tr>
</tbody>
</table>

Table 2

In each group n=20
* These absolute alkali-soluble fluoride values have to be interpreted with caution and may be high because of technique error
Etch Depth Means and Standard Deviations (µm)

<table>
<thead>
<tr>
<th>Etch Depth</th>
<th>No Surface treatment</th>
<th>Toothbrushing</th>
<th>Deproteinizing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No F Group I</td>
<td>No F Group IV</td>
<td>No F Group VII</td>
</tr>
<tr>
<td></td>
<td>2% NaF Group II</td>
<td>2% NaF Group V</td>
<td>2% NaF Group VIII</td>
</tr>
<tr>
<td></td>
<td>1 ppm F Group III</td>
<td>1 ppm F Group VI</td>
<td>1 ppm F Group IX</td>
</tr>
<tr>
<td>15 sec etch depth (µm)</td>
<td>5.81 ± 1.20</td>
<td>6.03 ± 1.47</td>
<td>6.09 ± 1.42</td>
</tr>
<tr>
<td></td>
<td>5.45 ± 1.31</td>
<td>6.51 ± 2.14</td>
<td>6.54 ± 1.97</td>
</tr>
<tr>
<td></td>
<td>6.08 ± 1.15</td>
<td>5.94 ± 1.08</td>
<td>6.63 ± 1.19</td>
</tr>
<tr>
<td>30 sec etch depth (µm)</td>
<td>7.66 ± 3.93</td>
<td>6.76 ± 3.53</td>
<td>6.86 ± 3.75</td>
</tr>
<tr>
<td></td>
<td>6.05 ± 3.14</td>
<td>6.96 ± 3.72</td>
<td>6.65 ± 2.60</td>
</tr>
<tr>
<td></td>
<td>8.28 ± 3.02</td>
<td>7.63 ± 2.85</td>
<td>8.62 ± 3.16</td>
</tr>
<tr>
<td>60 sec etch depth (µm)</td>
<td>12.35 ± 5.99</td>
<td>14.21 ± 7.21</td>
<td>11.54 ± 7.37</td>
</tr>
<tr>
<td></td>
<td>10.81 ± 7.32</td>
<td>12.77 ± 7.83</td>
<td>13.92 ± 5.64</td>
</tr>
<tr>
<td></td>
<td>12.77 ± 6.02</td>
<td>11.66 ± 7.77</td>
<td>15.41 ± 7.14</td>
</tr>
</tbody>
</table>

Table 3

In each group n=20
### Group Coefficients of Variance

<table>
<thead>
<tr>
<th>Fluoride / Etch Depth</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
<th>Group VI</th>
<th>Group VII</th>
<th>Group VIII</th>
<th>Group IX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkali soluble fluoride (μg)</td>
<td>164.69</td>
<td>69.45</td>
<td>89.97</td>
<td>73.95</td>
<td>95.07</td>
<td>82.97</td>
<td>48.19</td>
<td>64.90</td>
<td>175.05</td>
</tr>
<tr>
<td>15 sec etch fluoride (ppm)</td>
<td>46.59</td>
<td>33.17</td>
<td>41.63</td>
<td>38.23</td>
<td>36.27</td>
<td>56.14</td>
<td>40.60</td>
<td>34.46</td>
<td>44.70</td>
</tr>
<tr>
<td>30 sec etch fluoride (ppm)</td>
<td>42.01</td>
<td>49.01</td>
<td>35.86</td>
<td>54.29</td>
<td>78.32</td>
<td>28.96</td>
<td>25.04</td>
<td>37.85</td>
<td>37.49</td>
</tr>
<tr>
<td>60 sec etch fluoride (ppm)</td>
<td>47.60</td>
<td>51.40</td>
<td>34.75</td>
<td>104.33</td>
<td>29.40</td>
<td>51.78</td>
<td>33.05</td>
<td>42.09</td>
<td>30.45</td>
</tr>
<tr>
<td>15 sec etch depth (μm)</td>
<td>20.71</td>
<td>24.10</td>
<td>18.83</td>
<td>24.38</td>
<td>32.92</td>
<td>18.16</td>
<td>23.33</td>
<td>30.14</td>
<td>17.97</td>
</tr>
<tr>
<td>30 sec etch depth (μm)</td>
<td>51.26</td>
<td>51.96</td>
<td>36.43</td>
<td>52.31</td>
<td>53.43</td>
<td>37.35</td>
<td>54.62</td>
<td>39.04</td>
<td>36.71</td>
</tr>
<tr>
<td>60 sec etch depth (μm)</td>
<td>48.51</td>
<td>67.73</td>
<td>47.19</td>
<td>50.72</td>
<td>61.35</td>
<td>66.62</td>
<td>63.90</td>
<td>40.50</td>
<td>46.33</td>
</tr>
</tbody>
</table>

Table 4
<table>
<thead>
<tr>
<th>Group Ranges</th>
<th>Fluoride / Etch Depth</th>
<th>Alkali Soluble Fluoride (µg)</th>
<th>15 sec etch fluoride (ppm)</th>
<th>30 sec etch fluoride (ppm)</th>
<th>60 sec etch fluoride (ppm)</th>
<th>15 sec etch depth (µm)</th>
<th>30 sec etch depth (µm)</th>
<th>60 sec etch depth (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>4.32</td>
<td>2718.13</td>
<td>2861.35</td>
<td>2928.41</td>
<td>3097.50</td>
<td>4.76</td>
<td>7.52</td>
<td>11.49</td>
</tr>
<tr>
<td>Group II</td>
<td>4.65</td>
<td>3445.04</td>
<td>3445.04</td>
<td>3445.04</td>
<td>3445.04</td>
<td>4.76</td>
<td>7.52</td>
<td>11.49</td>
</tr>
<tr>
<td>Group III</td>
<td>4.65</td>
<td>3851.73</td>
<td>3851.73</td>
<td>3851.73</td>
<td>3851.73</td>
<td>4.76</td>
<td>7.52</td>
<td>11.49</td>
</tr>
<tr>
<td>Group IV</td>
<td>4.89</td>
<td>3255.92</td>
<td>3255.92</td>
<td>3255.92</td>
<td>3255.92</td>
<td>4.76</td>
<td>7.52</td>
<td>11.49</td>
</tr>
<tr>
<td>Group V</td>
<td>4.89</td>
<td>5887.74</td>
<td>5887.74</td>
<td>5887.74</td>
<td>5887.74</td>
<td>4.76</td>
<td>7.52</td>
<td>11.49</td>
</tr>
<tr>
<td>Group VI</td>
<td>4.88</td>
<td>2241.01</td>
<td>2241.01</td>
<td>2241.01</td>
<td>2241.01</td>
<td>4.76</td>
<td>7.52</td>
<td>11.49</td>
</tr>
<tr>
<td>Group VII</td>
<td>4.88</td>
<td>1258.06</td>
<td>1258.06</td>
<td>1258.06</td>
<td>1258.06</td>
<td>4.76</td>
<td>7.52</td>
<td>11.49</td>
</tr>
<tr>
<td>Group VIII</td>
<td>4.88</td>
<td>1746.90</td>
<td>1746.90</td>
<td>1746.90</td>
<td>1746.90</td>
<td>4.76</td>
<td>7.52</td>
<td>11.49</td>
</tr>
<tr>
<td>Group IX</td>
<td>4.88</td>
<td>1060.22</td>
<td>1060.22</td>
<td>1060.22</td>
<td>1060.22</td>
<td>4.76</td>
<td>7.52</td>
<td>11.49</td>
</tr>
</tbody>
</table>

Table 5
Statistical Analysis (ANOVA) - Alkali-Soluble Fluoride (μg)

<table>
<thead>
<tr>
<th>Inter-Fluoride Treatment</th>
<th>Toothbrushing</th>
<th>Deproteinizing</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Surface treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I vs. Group II</td>
<td>S***††</td>
<td>Group VII vs. Group VIII - S**†</td>
</tr>
<tr>
<td>Group I vs. Group III</td>
<td>NS</td>
<td>Group VII vs. Group IX - NS</td>
</tr>
<tr>
<td>Group II vs. Group III</td>
<td>S***+++</td>
<td>Group VIII vs. Group IX - S*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inter-Surface Treatment</th>
<th>2% Sodium Fluoride</th>
<th>1 ppm Fluoride</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Fluoride treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I vs. Group IV</td>
<td>NS</td>
<td>Group III vs. Group VI - NS</td>
</tr>
<tr>
<td>Group I vs. Group VII</td>
<td>NS</td>
<td>Group III vs. Group IX - NS</td>
</tr>
<tr>
<td>Group IV vs. Group VII</td>
<td>NS</td>
<td>Group VI vs. Group IX - NS</td>
</tr>
</tbody>
</table>

Table 6
NS - Not Significant at p=0.05  S - Significant at p=0.05
* - Significant at p=0.05 (Fisher's PLSD)  ** - Significant at p=0.01 (Fisher's PLSD)
*** - Significant at p=0.001 (Fisher's PLSD)
† - Significant at p=0.05 (Scheffe F-test)  †† - Significant at p=0.01 (Scheffe F-test)
††† - Significant at p=0.001 (Scheffe F-test)
### Statistical Analysis (ANOVA) - 15 second etch Fluoride (ppm)

#### Inter-Fluoride Treatment

<table>
<thead>
<tr>
<th>No Surface treatment</th>
<th>Toothbrushing</th>
<th>Deproteinizing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I vs. Group II - NS</td>
<td>Group IV vs. Group V - NS</td>
<td>Group VII vs Group VIII-S***††</td>
</tr>
<tr>
<td>Group I vs. Group III - NS</td>
<td>Group IV vs. Group VI - NS</td>
<td>Group VII vs. Group IX - NS</td>
</tr>
<tr>
<td>Group II vs. Group III - NS</td>
<td>Group V vs. Group VI - NS</td>
<td>Group VIII vs. Group IX - S*</td>
</tr>
</tbody>
</table>

#### Inter-Surface Treatment

<table>
<thead>
<tr>
<th>No Fluoride treatment</th>
<th>2% Sodium Fluoride</th>
<th>1 ppm Fluoride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I vs. Group IV - NS</td>
<td>Group II vs. Group V - NS</td>
<td>Group III vs. Group VI - NS</td>
</tr>
<tr>
<td>Group I vs. Group VII - S*</td>
<td>Group II vs. Group VIII - NS</td>
<td>Group III vs. Group IX - NS</td>
</tr>
<tr>
<td>Group IV vs. Group VII - NS</td>
<td>Group V vs. Group VIII - NS</td>
<td>Group VI vs. Group IX - NS</td>
</tr>
</tbody>
</table>

**Table 7**

NS - Not Significant at p=0.05  
S - Significant at p=0.05  
* - Significant at p=0.05 (Fisher's PLSD)  
** - Significant at p=0.01 (Fisher's PLSD)  
*** - Significant at p=0.001 (Fisher's PLSD)  
† - Significant at p=0.05 (Scheffe F-test)  
†† - Significant at p=0.01 (Scheffe F-test)
<table>
<thead>
<tr>
<th>Inter-Fluoride Treatment</th>
<th>Toothbrushing</th>
<th>Deproteinizing</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Surface treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I vs. Group II</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Group I vs. Group III</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Group II vs. Group III</td>
<td>S**†</td>
<td></td>
</tr>
<tr>
<td>Group IV vs. Group V</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Group IV vs. Group VI</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Group V vs. Group VI</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Inter-Surface Treatment</th>
<th>1% Fluoride</th>
<th>2% Sodium Fluoride</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Fluoride treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I vs. Group IV</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Group I vs. Group V</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Group I vs. Group VI</td>
<td>S†</td>
<td></td>
</tr>
<tr>
<td>Group IV vs. Group VII</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Group IV vs. Group VIII</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Group V vs. Group VIII</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

Table 8

- NS: Not Significant at p=0.05
- S: Significant at p=0.05 (Fisher's LSD)
- **†: Significant at p=0.01 (Fisher's LSD)
- ††: Significant at p=0.01 (Schiff's F-test)
### Statistical Analysis (ANOVA) - 60 second etch Fluoride (ppm)

<table>
<thead>
<tr>
<th></th>
<th>Inter-Fluoride Treatment</th>
<th>Inter-Surface Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Surface treatment</td>
<td>Toothbrushing</td>
</tr>
<tr>
<td>Group I vs. Group II</td>
<td>NS</td>
<td>Group IV vs. Group V</td>
</tr>
<tr>
<td>Group I vs. Group III</td>
<td>NS</td>
<td>Group IV vs. Group VI</td>
</tr>
<tr>
<td>Group II vs. Group III</td>
<td>S*†</td>
<td>Group V vs. Group VI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Group VII vs. Group VIII</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Group VII vs. Group IX</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Group VIII vs. Group IX</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>No Fluoride treatment</th>
<th>2% Sodium Fluoride</th>
<th>1 ppm Fluoride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I vs. Group IV</td>
<td>NS</td>
<td>Group II vs. Group V</td>
<td>- S*</td>
</tr>
<tr>
<td>Group I vs. Group VII</td>
<td>NS</td>
<td>Group II vs. Group VIII</td>
<td>- NS</td>
</tr>
<tr>
<td>Group IV vs. Group VII</td>
<td>NS</td>
<td>Group V vs. Group VIII</td>
<td>- NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Group III vs. Group VI</td>
<td>- S*†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Group III vs. Group IX</td>
<td>- NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Group VI vs. Group IX</td>
<td>- S*</td>
</tr>
</tbody>
</table>

**Table 9**

NS - Not Significant at p=0.05    S - Significant at p=0.05

* - Significant at p=0.05 (Fisher's PLSD)

† - Significant at p=0.05 (Scheffe F-test)
### Statistical Analysis (ANOVA) - 15 second Etch Depth (µm)

#### Inter-Fluoride Treatment

<table>
<thead>
<tr>
<th>No Surface treatment</th>
<th>Toothbrushing</th>
<th>Deproteinizing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I vs. Group II</td>
<td>- NS</td>
<td>Group VII vs. Group VIII</td>
</tr>
<tr>
<td>Group I vs. Group III</td>
<td>- NS</td>
<td>Group VII vs. Group IX</td>
</tr>
<tr>
<td>Group II vs. Group III</td>
<td>- NS</td>
<td>Group VIII vs. Group IX</td>
</tr>
</tbody>
</table>

#### Inter-Surface Treatment

<table>
<thead>
<tr>
<th>No Fluoride treatment</th>
<th>2% Sodium Fluoride</th>
<th>1 ppm Fluoride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I vs. Group IV</td>
<td>- NS</td>
<td>Group III vs. Group VI</td>
</tr>
<tr>
<td>Group I vs. Group VII</td>
<td>- NS</td>
<td>Group III vs. Group IX</td>
</tr>
<tr>
<td>Group IV vs. Group VII</td>
<td>- NS</td>
<td>Group VI vs. Group IX</td>
</tr>
</tbody>
</table>

Table 10

NS - Not Significant at p=0.05  
S - Significant at p=0.05  
* - Significant at p=0.05 (Fisher's PLSD)
### Statistical Analysis (ANOVA) - 30 second Etch Depth (\(\mu m\))

#### Inter-Fluoride Treatment

<table>
<thead>
<tr>
<th>No Surface treatment</th>
<th>Toothbrushing</th>
<th>Deproteinizing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I vs. Group II - NS</td>
<td>Group IV vs. Group V - NS</td>
<td>Group VII vs. Group VIII - NS</td>
</tr>
<tr>
<td>Group I vs. Group III - NS</td>
<td>Group IV vs. Group VI - NS</td>
<td>Group VII vs. Group IX - NS</td>
</tr>
<tr>
<td>Group II vs. Group III - NS</td>
<td>Group V vs. Group VI - NS</td>
<td>Group VIII vs. Group IX - NS</td>
</tr>
</tbody>
</table>

#### Inter-Surface Treatment

<table>
<thead>
<tr>
<th>No Fluoride treatment</th>
<th>2% Sodium Fluoride</th>
<th>1 ppm Fluoride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I vs. Group IV - NS</td>
<td>Group II vs. Group V - NS</td>
<td>Group III vs. Group VI - NS</td>
</tr>
<tr>
<td>Group I vs. Group VII - NS</td>
<td>Group II vs. Group VIII - NS</td>
<td>Group III vs. Group IX - NS</td>
</tr>
<tr>
<td>Group IV vs. Group VII - NS</td>
<td>Group V vs. Group VIII - NS</td>
<td>Group VI vs. Group IX - NS</td>
</tr>
</tbody>
</table>

Table 11

NS - Not Significant at p=0.05
**Statistical Analysis (ANOVA) - 60 second Etch Depth (μm)**

<table>
<thead>
<tr>
<th></th>
<th>No Surface treatment</th>
<th>Toothbrushing</th>
<th>Deproteinizing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I vs. Group II</td>
<td>- NS</td>
<td>Group IV vs. Group V - NS</td>
<td>Group VII vs. Group VIII - NS</td>
</tr>
<tr>
<td>Group I vs. Group III</td>
<td>- NS</td>
<td>Group IV vs. Group VI - NS</td>
<td>Group VII vs. Group IX - NS</td>
</tr>
<tr>
<td>Group II vs. Group III</td>
<td>- NS</td>
<td>Group V vs. Group VI - NS</td>
<td>Group VIII vs. Group IX - NS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>No Fluoride treatment</th>
<th>2% Sodium Fluoride</th>
<th>1 ppm Fluoride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I vs. Group IV</td>
<td>- NS</td>
<td>Group II vs. Group V - NS</td>
<td>Group III vs. Group VI - NS</td>
</tr>
<tr>
<td>Group I vs. Group VII</td>
<td>- NS</td>
<td>Group II vs. Group VIII - NS</td>
<td>Group III vs. Group IX - NS</td>
</tr>
<tr>
<td>Group IV vs. Group VII</td>
<td>- NS</td>
<td>Group V vs. Group VIII - NS</td>
<td>Group VI vs. Group IX - NS</td>
</tr>
</tbody>
</table>

Table 12

NS - Not Significant at p=0.05
Statistical Analysis (ANOVA) - Group Intra-tooth Comparisons

<table>
<thead>
<tr>
<th>Fluoride / Etch Depth</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
<th>Group IV</th>
<th>Group V</th>
<th>Group VI</th>
<th>Group VII</th>
<th>Group VIII</th>
<th>Group IX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkali soluble fluoride (μg)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>15 sec etch fluoride (ppm)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>30 sec etch fluoride (ppm)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>60 sec etch fluoride (ppm)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>S*†</td>
</tr>
<tr>
<td>15 sec etch depth (μm)</td>
<td>S*†</td>
<td>S*†</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>30 sec etch depth (μm)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>60 sec etch depth (μm)</td>
<td>NS</td>
<td>NS</td>
<td>S*†</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 13

NS - Not Significant at p=0.05   S - Significant at p=0.05
* - Significant at p=0.05 (Fisher's PLSD)
† - Significant at p=0.05 (Scheffe F-test)
Fig. 1. Unerupted third molar (stored for one day) stained with toluidine blue a) without toothbrushing  b) with toothbrushing
Fig. 2. Unerupted third molar (stored for 21 days) stained
with toluidine blue  a) without deproteinizing  b) with
deproteinizing
Fig. 3. SEM of unerupted third molar with no surface fluoride treatment  
   a) without KOH wash (X 1000)  
   b) with KOH wash (X 1000)
Fig. 4. SEM of unerupted third molar with toothbrushing surface treatment and no fluoride application
a) without KOH wash (X 1000)  b) with KOH wash (X 1000)
Fig. 5. SEM of unerupted third molar with deproteinizing surface treatment and no fluoride application
a) without KOH wash (X 1000)  b) with KOH wash (X 1000)
Fig. 6. SEM of unerupted third molar with no surface treatment and 2% NaF (9048 ppm F⁻) application
   a) without KOH wash (X 1000)  b) without KOH wash (X 5000)  c) with KOH wash (X 1000)
Fig. 7. SEM of unerupted third molar with toothbrushing surface treatment and 2% NaF (9048 ppm F) application
a) without KOH wash (X 1000) b) with KOH wash (X 1000)
Fig. 8. SEM of unerupted third molar with deproteinizing surface treatment and 2% NaF (9048 ppm F⁻) application
a) without KOH wash (X 1000) b) with KOH wash (X 1000)
Fig. 9. SEM of unerupted third molar with no surface
treatment and 1 ppm fluoride application  a) without
KOH wash (X 1000)  b) with KOH wash (X 1000)
Fig. 10. SEM of unerupted third molar with toothbrushing surface treatment and 1 ppm fluoride application
a) without KOH wash (X 1000) b) with KOH wash (X 1000)
Fig. 11. SEM of unerupted third molar with deproteinizing surface treatment and 1 ppm fluoride application
a) without KOH wash (X 1000) b) with KOH wash (X 1000)
Appendix 1

Random Allocation of Teeth in Part I

Total number of teeth = 90

Random sequence (Petrie, 1978):

| 23 | 01 | 71 | 61 | 37 | 30 | 22 | 70 | 87 | 90 | - Group I |
| 76 | 31 | 69 | 17 | 66 | 50 | 09 | 39 | 49 | 19 | - Group II |
| 68 | 07 | 08 | 54 | 20 | 73 | 16 | 32 | 60 | 26 | - Group III |
| 04 | 43 | 11 | 13 | 63 | 24 | 47 | 74 | 65 | 67 | - Group IV |
| 62 | 57 | 88 | 45 | 75 | 41 | 35 | 27 | 64 | 15 | - Group V |
| 29 | 44 | 48 | 58 | 42 | 03 | 81 | 89 | 79 | 46 | - Group VI |
| 18 | 06 | 82 | 25 | 28 | 53 | 80 | 55 | 40 | 78 | - Group VII |
| 05 | 59 | 33 | 34 | 12 | 10 | 02 | 51 | 38 | 36 | - Group VIII |
| 83 | 21 | 84 | 77 | 56 | 86 | 14 | 85 | 52 | 72 | - Group IX |

Allocation of teeth: (Tooth number - Group number)

| 1) I | 19) II | 37) I | 55) VII | 73) III |
| 2) VIII | 20) III | 38) VIII | 56) IX | 74) IV |
| 3) VI | 21) IX | 39) II | 57) V | 75) V |
| 4) IV | 22) I | 40) VII | 58) VI | 76) II |
| 5) VIII | 23) I | 41) V | 59) VIII | 77) IX |
| 6) VII | 24) IV | 42) VI | 60) III | 78) VII |
| 7) III | 25) VII | 43) IV | 61) I | 79) VI |
| 8) III | 26) III | 44) VI | 62) V | 80) VII |
| 9) II | 27) V | 45) V | 63) IV | 81) VI |
| 10) VIII | 28) VII | 46) VI | 64) V | 82) VII |
| 11) IV | 29) VI | 47) IV | 65) IV | 83) IX |
| 12) VIII | 30) I | 48) VI | 66) II | 84) IX |
| 13) IV | 31) II | 49) II | 67) IV | 85) IX |
| 14) IX | 32) III | 50) II | 68) III | 86) IX |
| 15) V | 33) VIII | 51) VIII | 69) II | 87) I |
| 16) III | 34) VIII | 52) IX | 70) I | 88) V |
| 17) II | 35) V | 53) VII | 71) I | 89) VI |
| 18) VII | 36) VIII | 54) III | 72) IX | 90) I |
Appendix 2

Random Allocation of Teeth in Part II

Total number of teeth = 18

Random sequence (Petrie, 1978):

13 14 - Group I
06 01 - Group II
04 03 - Group III
10 11 - Group IV
02 12 - Group V
09 05 - Group VI
16 18 - Group VII
08 15 - Group VIII
17 07 - Group IX

Allocation of teeth: (Tooth number - Group number)

1) II 10) IV
2) V 11) IV
3) III 12) V
4) III 13) I
5) VI 14) I
6) II 15) VIII
7) IX 16) VII
8) VIII 17) IX
9) VI 18) VII
Appendix 3

Formulae for Chemical Analytic Calculations

Alkali-Soluble Fluoride Analysis: The weight of fluoride was determined from fluoride ion concentration using the plot of millivoltage potentials of standard fluoride solutions.

Volume of alkali (1M KOH) treatment solution = 10.0 ml
2.0 ml of this solution was used for analysis.
Therefore, dilution factor = 5 (DF-1)
2.0 ml of low level Tisab solution was added to 2.0 ml of alkali treatment solution as a buffer. Then 2.8 or 2.7 ml of 1M HNO₃ was added to titrate the solution to a pH of 5.2-5.6 so that fluoride ion measurements could be done with the electrode.
Therefore, dilution factor = 3.4 or 3.35 (DF-2)
Therefore, total dilution factor = 17.0 or 16.75
Alkali-soluble fluoride weight (μg) = Fluoride reading x 17.0/16.75

Bound Fluoride Analysis: The weight of fluoride was determined from fluoride ion concentration using the plot of millivoltage potentials of standard fluoride solutions.

Fluoride weight (μg):

Fluoride reading = ppm F (μg/ml)
Volume of etch solution = 0.5 ml
Volume of Tisab solution = 0.5 ml
Therefore, dilution factor = 2
Fluoride weight (μg) = Fluoride reading x 2

Phosphorous weight (μg):

Phosphorous reading = ppm P (μg/ml)
Volume of etch solution = 0.5 ml
Volume of Tisab solution = 0.5 ml
Therefore, dilution factor = 2 (DF-1)
0.2 ml of etch + Tisab mix solution was used for P analysis.
Therefore, dilution factor = 5 (DF-2)
Therefore, total dilution factor = 10
Phosphorous weight (μg) = Phosphorous reading x 10
Enamel weight (µg):

Concentration of Phosphorous in enamel is 18.1% (Angmar et al., 1963).
Enamel weight (µg) = \( \text{Phosphorous weight (µg)} \div 0.181 \)

ppm F⁻ in tooth:

\[
\text{ppm } F^- \text{ in tooth} = \frac{\text{Fluoride weight (µg)} \times 10^6}{\text{Enamel weight (µg)}}
\] (Arends and Schuthof, 1975)

Volume of enamel:

Density of enamel is 2.95 g/cm³ (Manly et al., 1939).

\[
\text{Volume of enamel} = \frac{\text{Enamel weight (µg)}}{2.95 \times 10^6}
\]

Etch Depth Analysis: Area exposed = 10.65 mm² = .1065 cm²

\[
\text{Etch depth (cm)} = \frac{\text{Volume of enamel (cm³)}}{\text{Area exposed (cm²)}}
\]

= \text{Volume of enamel} cm

\[ \div .1065 \]
Appendix 4

Pilot Study I

Objectives:
1. To stain and identify an organic layer on enamel surface of fully formed pre-eruptively mature third molars.
2. To stain for the presence/absence of the organic layer following storage for different intervals of time.
3. To stain for the presence/absence of the organic layer following various surface treatments.

Materials and Methods:

Enamel samples: Human unerupted third molars with more than half their roots formed were used.

Storage of teeth: The surgically removed third molars were stored at 4°C on cotton soaked with distilled water containing thymol crystals.

Duration of storage: Teeth were stored for:
   a) one day
   b) four days
   c) twentyone days

Staining of teeth: The teeth were stained for one minute in a 10 ml solution of the stain in a plastic bottle. Stains employed were:
   a) Hematoxylin
   b) Toluidine blue

Surface treatment of teeth: The teeth received either a toothbrushing or deproteinizing surface treatment.

Toothbrushing treatment: One surface of the tooth received a one minute brushing with a soft toothbrush and deionized water.

Deproteinizing treatment: Each tooth was partially immersed in a 0.1M Urea solution in a plastic bottle for seven days at 37°C. The solution was not stirred.

Experimental design: There were twelve experimental groups:
1) One day storage; toluidine blue stain; toothbrushing treatment
2) One day storage; hematoxylin stain; toothbrushing treatment
3) One day storage; toluidine blue stain; deproteinizing treatment
4) One day storage; hematoxylin stain; deproteinizing treatment
5) Four days storage; toluidine blue stain; toothbrushing treatment
6) Four days storage; hematoxylin stain; toothbrushing treatment
7) Four days storage; toluidine blue stain; deproteinizing treatment
8) Four days storage; hematoxylin stain; deproteinizing treatment
9) Twentyone days storage; toluidine blue stain; toothbrushing treatment
10) Twentyone days storage; hematoxylin stain; toothbrushing treatment
11) Twentyone days storage; toluidine blue stain; deproteinizing treatment
12) Twentyone days storage; hematoxylin stain; deproteinizing treatment

Two teeth were randomly allocated to each group. The teeth were rinsed in a stream of 50 ml of deionized water and then stained. After staining, the teeth were again rinsed in a stream of 50 ml of deionized water. The teeth were then photographed. Then, the teeth were treated with either toothbrushing or deproteinizing surface treatment followed by a rinse in a stream of 50 ml of deionized water and again stained. After staining, the teeth were rinsed in a stream of 50 ml of deionized water and again photographed.

Results:
1. An organic layer of variable extent was present on the enamel surface of fully formed pre-eruptively mature third molars. This organic layer was well stained by toluidine blue rather than hematoxylin.
2. The organic layer was present on teeth even after twentyone days of storage at 4°C on cotton soaked with deionized water containing thymol crystals.
3. The organic layer was removed by both toothbrushing as well as deproteinizing surface treatments.
Appendix 5

Pilot Study II

Objective: To determine the standardization of enamel biopsy area using an adhesive tape with a window.

Material and Methods:

Enamel samples: Human erupted third molars were used.

Storage of teeth: The surgically removed third molars were stored at 4°C on cotton soaked with distilled water containing thymol crystals.

Number of teeth: Four teeth were used.

Preparation of specimens: The teeth were pumice prophylaxed and the roots cut off. The teeth were then sectioned into two parts. One half from each tooth was allocated to each experimental group.

Experimental groups: There were two experimental groups:
I. Enamel biopsy area standardized using a adhesive tape with a window (10.65 mm²) and yellow sticky wax.
II. Enamel biopsy area standardized using adhesive circle of 10.65 mm² (removed prior to biopsy) and yellow sticky wax.

Topical fluoride treatment: Each specimen received a four minute treatment with 10 ml of aqueous non-buffered 2% NaF (9048 ppm F⁻) solution in a glass beaker. Following the fluoride treatment, the teeth were rinsed in 200 ml of distilled water for two minutes.

Experimental design: Group II specimens had the enamel biopsy area standardized prior to fluoride treatment. All specimens in Groups I and II received the topical fluoride treatment. Following fluoride treatment and distilled water rinse, all Group I specimens had the enamel biopsy area standardized. The specimens were then assayed for fluoride.

Fluoride assay: Each specimen was washed in 1M KOH (10 ml) solution for 24 hours to remove the alkali-soluble fluoride. Following the KOH wash, the specimens were further assayed for bound fluoride by acid etching. The specimens received 15-, 30- and 60- second exposures of 0.5M Perchloric acid (0.5 ml) followed by a rinse in low-level Tisab (0.5 ml). Fluoride estimations were made as described (Appendix 3).
Statistical analysis: Mean group values of the various parameters were obtained and compared by paired t-test at p=0.1 (90%).

Results:

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkali-soluble fluoride (μg)</td>
<td>0.636</td>
<td>0.352</td>
</tr>
<tr>
<td>15 sec- etch fluoride (ppm)</td>
<td>2662.23</td>
<td>2886.11</td>
</tr>
<tr>
<td>30 sec- etch fluoride (ppm)</td>
<td>1518.47</td>
<td>1548.20</td>
</tr>
<tr>
<td>60 sec- etch fluoride (ppm)</td>
<td>931.00</td>
<td>1046.41</td>
</tr>
<tr>
<td>15 sec- etch depth (μm)</td>
<td>6.57</td>
<td>3.27</td>
</tr>
<tr>
<td>30 sec- etch depth (μm)</td>
<td>10.87</td>
<td>8.05</td>
</tr>
<tr>
<td>60 sec- etch depth (μm)</td>
<td>20.87</td>
<td>18.62</td>
</tr>
</tbody>
</table>

Alkali-soluble fluoride levels were significantly greater in Group I. Bound fluoride levels as well as etch depths showed no significant differences between the two groups.

Conclusions: The adhesive tape with window used for standardizing biopsy area lost some adhesiveness along the window margins due to prolonged exposure in KOH solution. This affects absolute alkali-soluble fluoride measurements. However, the bound fluoride analysis was not affected probably due to short duration of exposure to etch solution.
Bibliography


32. Chase SW; The origin, structure and duration of Nasmyth's membrane. Anat Rec (1926) 33:357-76.


