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DE- AND RE-MINERALISATION OF HUMAN DENTAL ENAMEL

USING SINGLE SECTIONS

By

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DECLARATION

This thesis is the original work of the author.

Frankanne Damato.

Summary

Optimal fluoride treatment regimes have not yet been established, despite many years of investigation. Accordingly, the main aims of this thesis were (i) to develop an *in vitro* pH cycling model to investigate the effects of fluoride concentration on de-/ remineralisation and (ii) to further develop an *in situ* model for studying these factors in the natural oral environment.

Microradiography and computerised microdensitometry were the techniques employed for assessing mineral content in human dental enamel sections. The first study showed no significant differences in demineralisation between bulk and thin sections of enamel. This was important as the ability to use thin enamel sections in de-/ remineralisation studies enables the mineral content to be measured, before, during and after the experiment, thereby overcoming the problem of inhomogeneity and enabling small changes in mineral content to be accurately assessed.

Many laboratory systems are available for producing artificial subsurface carious lesions. In a comparative study, it was shown that solution-prepared artificial carious lesions were more responsive to de-/ remineralisation processes than lesions prepared by a gelatin system and therefore better suited for studies incorporating the use of fluoride. For this reason,

calcium phosphate solutions were used throughout this project to prepare subsurface lesions and to simulate de-/ remineralisation conditions.

Current knowledge suggests that fluoride must be present in the aqueous phase to inhibit demineralisation and to enhance remineralisation. A pH cycling study incorporating pre-formed artificial carious lesions was employed to mimic the elevated baseline salivary fluoride levels which are known to be present in the mouth with frequent use of a fluoride agent (eg. pastes or rinses). The results showed that the pre-formed lesions responded significantly to increase in fluoride concentration in the remineralising solutions thus demonstrating the importance of frequency of fluoride application.

The importance of the transient high fluoride levels, present in the mouth immediately after topical fluoride application were also investigated using *in vitro* pH cycling. A daily five minute exposure of the enamel specimens to neutral sodium fluoride solutions of different concentrations resulted in net remineralisation. However, solutions containing fluoride concentrations greater than 500 ppm did not produce any further significant increase in remineralisation, thereby indicating an optimium fluoride concentration at around 500 ppm.

Much attention has recently been given to *in situ* studies

which provide a natural environment for the study of caries preventive substances. The sensitivity of an *in situ* model was improved by using solution-prepared lesions which are more responsive than gelatin-prepared lesions, and by increasing the number of volunteers. This superior study allowed a fluoride dose-response relationship with sodium monofluorophosphate dentifrices to be demonstrated. In addition, significant differences between the two sides of the mouth could be measured as well as marked variations between volunteers in response to fluoridated and non-fluoridated pastes.

The *in vitro* and *in situ* studies described in this project should be useful in complementing results from caries clinical trials which are the ultimate means of validating caries-preventive treatments.

Chapter 1

Introduction

1.1 Tooth structure

The mineralised tissues of the human tooth are enamel, dentine and cementum. Enamel has a thickness of 1 - 3 mm and covers that part of the tooth exposed to the oral cavity. It is the most dense of all biological tissues with a density approaching 3 $kg.m^{-3}$ and consists of 96 % by weight mineral, 3 % by weight water and 1 % by weight organic matter (Jenkins, 1978). Enamel is avascular and acellular and is secreted from cells called ameloblasts, which are derived from the ectoderm. During formation, vesicles inside the ameloblasts aggregate and fuse with the cell membrane which then ruptures and the contents become extracellular. This discharged material is the organic matrix of the first formed enamel. During this deposition, needle-like crystals of hydroxyapatite appear within it. These crystallites are arranged in structures called prisms (about 5 µm in diameter) running from the amelodentinal junction to the tooth surface. In cross-section these prisms are often keyhole-shaped in appearance. The crystallites (about 40 nm in diameter) are densely packed, and those central ones are orientated roughly parallel to the axis of the prism, whereas at the prism borders the crystallites are less dense and placed more at random. The spaces between prisms are thought to

be filled mainly with water and organic matter (Arends & ten Cate, 1981). During enamel maturation the crystals increase in thickness and become more ordered. As more inorganic ions are incorporated, protein and water are lost. Prior to influx of mineral, amelogenin proteins are almost completely removed from the enamel, whereas the enamelins (acidic glycoproteins), which are tightly bound to crystallites, comprise most of the 1 % residual protein found in mature enamel (Osborne & ten Cate, 1983).

Each unit cell of the hydroxyapatite crystal consists of ten calcium ions, six phosphate ions and two hydroxyl ions. Its stoichiometric formula is thus $Ca_{10}(PO_4)_6(OH)_2$ The unit cell is screw-hexagonal with its a - and b - axes interesting at 120° and equal in length (Einspahr & Bugg, 1980). Enamel is an imperfect apatite, low in calcium and hydroxide ions but rich in substitutional ions (Eanes, 1979) including carbonate, sodium, magnesium, chloride and fluoride ions. The fluoride and chloride ions are found at higher concentrations near the surface whereas the other ions are more concentrated near the amelodentinal junction (Zipkin, 1970). The lower calcium to phosphate ratio has been attributed, among other factors to the presence (Eanes, 1979), of 5 % acid phosphate (HPO_4^{-2}) which, having lower charge, allows (e.g. example sodium or potassium) substitution for calcium in the lattice, while maintaining charge neutrality.

Neutron diffraction data (Kay, Young & Posner, 1964) support the hypothesis that the hydroxyl ions in the crystal lattice are arranged in a disordered column with the direction of the hydroxyls in the column reversed at various places. As a result of this disorder there are, occasionally, voids or vacancies along the length of a crystal because there is insufficient space to accommodate the hydroxyl ions. Young and Elliot (1966) suggested that fluoride provided a site for reversal of the polar hydroxyls. The fluoride ion can fit perfectly in the centre of the triangular arrangement of calcium ions, replacing one hydroxyl group, and stabilizing the crystal structure.

When the tooth erupts into the oral cavity it is exposed to a dynamic environment. Enamel, although not a living tissue, takes part in reactions which include: (1) solute ion transport from saliva to dentine; (2) ion exchange reactions with saliva including de- and remineralisation. (Einspahr & Bugg,1980).

1.2 Enamel caries

1.2.1 Introduction

Dental caries is a process which involves the progressive destruction of dental enamel by dissolution of hydroxyapatite mineral from the outer surface towards the dentine (Robinson, Weatherell & Hallsworth, 1983). In man, caries is commonly considered a chronic infectious

disease introduced into Europe during the neolithic period. The aetiology of dental caries has been investigated for many centuries. Guy de Chauliac (1300-1368) supported the theory that caries was caused by worms. The vital theory, which regarded caries as originating from within the tooth, was the most widely accepted until the middle of the nineteeth century.

The chemical theory of Parmly (1819), and the parasitic theory of Erdl (1843) were advanced by Miller (1890) to give the chemoparasitic theory, now almost universally accepted. This theory states that caries is caused by the action of acids on the mineral component of enamel. Miller's theory was substantiated by Pasteur's discovery that microorganisms transform sugars to lactic acids and by the *in vitro* work of Magitot (1867) and Leber & Rottenstein (1867), who showed similar findings. Today, caries is best described as a multifactorial disease in which there is an interplay of three principal factors: (1) the host (saliva and teeth); (2) the microflora; (3) the substrate or diet. In addition, adequate time must elapse with the three conditions interacting to permit transition from a sound surface to a carious one.

1.2.2 Histology of early lesions

On smooth enamel surfaces, the earliest visible sign of caries is loss of transparency resulting in an opaque chalky region - "the white spot" (Fig. 1.1). The

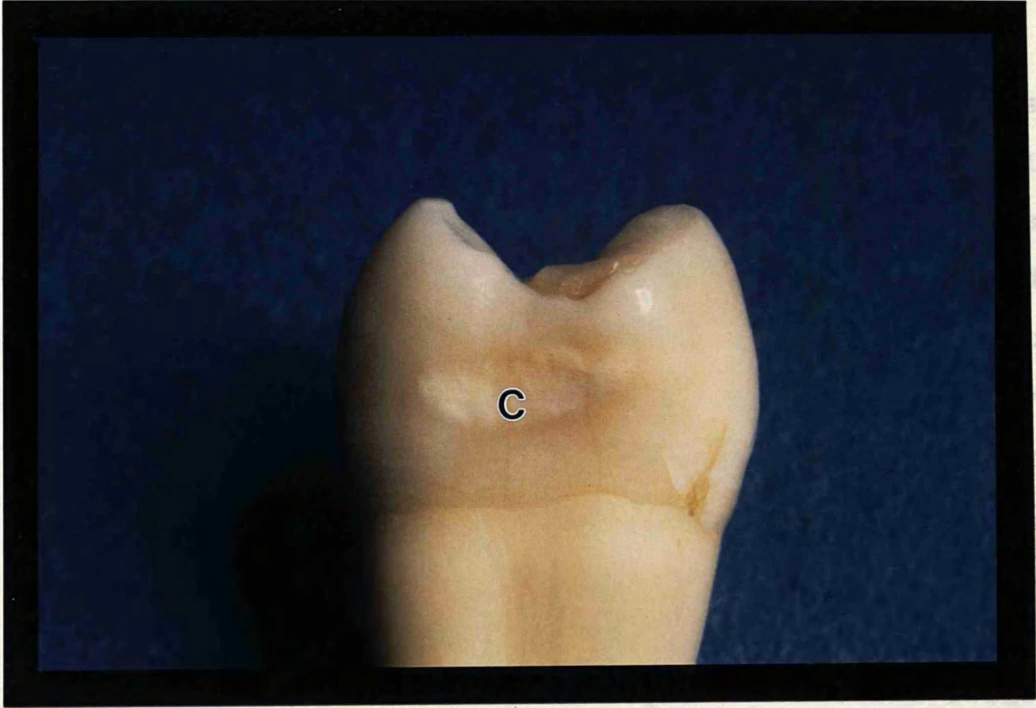


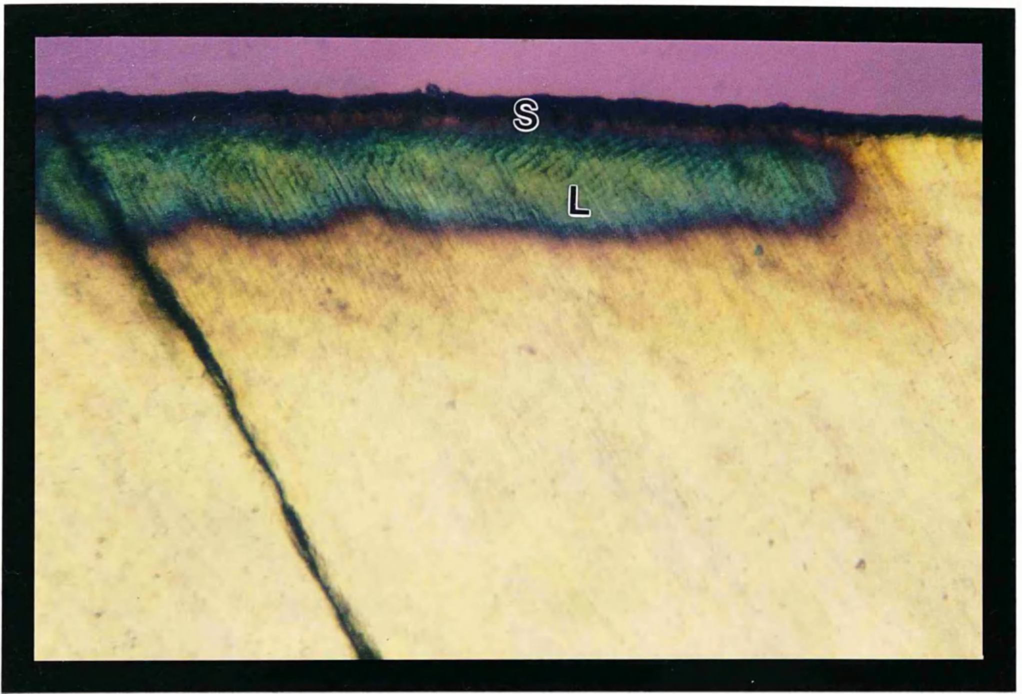
Figure 1.1 **Human premolar tooth with natural interproximal enamel caries (C)**

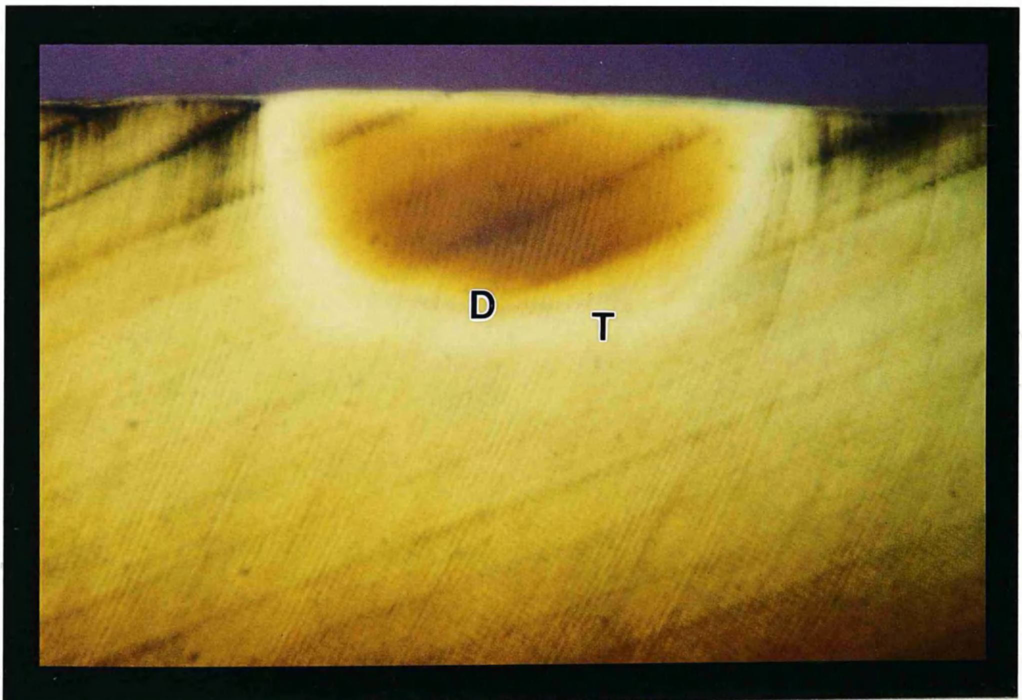
morphology of a carious lesion has been well described by Nishimura (1926), Darling (1956), Gustafson (1957) and others, and consists of an apparently sound outer layer of enamel at the surface, below which is a demineralised region.

Polarised light microscopy divides the carious lesion into four clearly distinguishable zones (Fig. 1.2): (1) a translucent zone which indicates the earliest changes in enamel at the advancing front of the lesion; (2) a dark zone varying considerably in width and showing a reduction in mineral of about 6 % per unit volume; (3) the body of the lesion which is the largest zone and the lowest in mineral content and, (4) the surface zone which can be up to 100 μm in thickness. The loss of mineral in this zone is variable, Bergman and Lind (1966) reporting a loss of 8 % volume mineral in the surface layer of natural lesions, although, a mineral loss in the range of 20 % by volume was not uncommon in the artificial lesions used throughout the present study. Scanning electron microscopy of surface zones of natural carious lesions revealed openings, eroded "focal holes" and Tomes process pits (Holma, Granath & Gustafson, 1970). "Focal holes" have also been observed in intact enamel. Another characteristic of this zone is the inclusion of larger and more perfect crystals associated with a high surface fluoride concentration.

Figure 1.2a Photomicrograph of a longitudinal ground section through an artificial lesion of enamel caries examined in water with polarized light to show the surface zone (S), and the the lesion body (L)

Figure 1.2b Photomicrograph of a longitudinal ground section through an artificial lesion of enamel caries examined in quinoline with polarized light to show the dark zone (D) and the translucent zone (T)


S
L


D
T

1.2.3 Mechanisms for subsurface demineralisation

The formation of dental caries is more complex than a simple dissolution of the hard tissue (Moreno & Zahradnik, 1979) caused by the hydroxonium ion attacking the crystal structure of the enamel apatite. What has intrigued many workers is the fact that the surface retains a high mineral content giving rise to subsurface demineralisation and that the dissolution proceeds locally rather than as a general dissolution of enamel. Recently it has been shown (Margolis & Moreno, 1985; Arends & Christoffersen, 1986) that "surface softening" (ie. the earliest stages of enamel demineralisation in which enamel is removed interprismatically) precedes surface layer formation. Some workers (Koulourides, Feagin & Pigman, 1965; Ogaard, 1985; Dijkmann, Schuthof & Arends, 1986; Corpron *et al.*, 1986) have worked with surface softened enamel in the hope of obtaining better information on the mechanisms of de-/ remineralisation.

There have been several attempts to explain the phenomenon of subsurface demineralisation. Von Bartheld (1961) suggested that the enamel surface exhibits a membrane potential and that enamel caries resulted from an ionic distribution of the Donnan type dependant on the presence and concentration of the proteins and derivatives of dental plaque, rather than the presence of free acid. Other workers (Holly & Gray, 1968; Francis & Briner, 1973) claimed that the outer surface of enamel became insoluble due to the effects of protective agents. The agents suggested were fluoride, salivary proteins, organic

polymers and poly - and diphosphonates. In this model the reactants, the hydroxonium ion and the undissociated acid, are diffusing to the reaction sites deep into the enamel, followed by a heterogeneous reaction, the products of which subsequently diffuse out of the enamel. Van Dijk and co-workers, (1979) attributed subsurface demineralisation to anatomical features. From computer simulations, these authors concluded that the presence of the surface layer could be explained by assuming gradients in the porosity, solubility and dissolution rates. Margolis and Moreno (1985) have suggested a model based on a dissolution-precipitation mechanism. Here the hydroxonium ion and the undissociated acid are said to diffuse into enamel and cause a series of phase transitions which result in dicalcium phosphate dihydrate and fluorapatite in the surface enamel. The model suggests a flow of basic ingredients from the inner enamel to the surface layer - a reprecipitation process. Anderson and Elliot (1987) believed that subsurface demineralisation was a general feature of the acidic dissolution of porous solids. They applied the theory of coupled ionic diffusion of Onsager & Fuoss (Tyrell & Harris, 1984) to explain the formation of subsurface demineralisation.

Work carried out on synthetic hydroxyapatite (Langdon, Elliot & Fearnhead, 1980; Anderson & Elliot, 1985) has shown that anatomical features (ie. orientation of the crystals and the presence of an organic matrix), as well

as the presence of chemical gradients of certain ions, were not necessary for subsurface demineralisation. Subsurface demineralisation has been successfully produced on human enamel without the use of inhibitors (Besic, 1953; Coolidge, Besic & Jacobs 1955; Theuns *et al.*, 1983; 1984a, 1984b; Theuns, Driessens & Van Dijk, 1986), thereby showing these were not essential for subsurface demineralisation.

Although the above theories, and others reviewed by Arends and Christoffersen (1986), have contributed to a better understanding of how the surface layer is formed or retained during a carious attack, there is still no generally accepted explanation. Nonetheless the ease with which subsurface demineralisation can be created *in vitro* has helped workers to obtain information, not only on caries formation, but also on remineralisation processes and on the anticaries efficacy of fluoride agents.

1.3 Remineralisation

Remineralisation is the deposition of mineral in carious enamel. This repairing process requires the penetration of ionic species, namely calcium and phosphate, from saliva or calcifying solutions, into the bulk of the lesion. Surface precipitation is not remineralisation (Moreno & Zahradnik, 1979). In a review of remineralisation (Arends & ten Cate, 1981), the term "remineralisation" is used for the deposition of calcium

phosphates in, or on dental enamel after a caries attack, acid softening or acid etching. In this thesis, remineralisation will be referred to as the deposition of mineral in the surface layer and / or in the body of a typical subsurface lesion.

There is a delicate balance in the oral cavity between demineralisation and remineralisation. The remineralising potential of saliva was first demonstrated by Head (1912). Saliva is secreted by the major and minor salivary glands and consists of 0.6 % solid matter, 0.3 % organic matter, 0.3 % inorganic matter and 98.8 % water. Saliva has many functions, among the most important being oral lubrication and the maintenance of tooth integrity. The latter is achieved by (1) the remineralising potential of its inorganic phase, and (2) the buffering systems present in saliva, the most important being the the carbonic acid / bicarbonate system.

The inorganic phase of saliva consists mainly of potassium sodium, chloride, bicarbonate, calcium, phosphate and magnesium (Ericson, 1959). The main factors governing the stability of the enamel apatites in saliva are the pH and the concentrations of calcium, phosphate and fluoride in solution. Thus the product of the activities of these ions in the liquid phase will determine whether or not dissolution of enamel takes place. The ion activity product of hydroxyapatite (HAP)

and fluorapatite (FAP) in saliva is expressed as follows:

For (HAP) $Ip = (Ca^{+2})^{10}.(PO_4{}^{-3})^6.(OH^-)^2$

For (FAP) $Ip = (Ca^{+2})^{10}.(PO_4{}^{-3})^6.(F^-)^2$

At the state of equilibrium this ion activity product is maximium and referred to as the thermodynamic solubility product.

Thus $Ip = Ksp$ where Ksp = solubility product

$Ip > Ksp$ supersaturation conditions

$Ip < Ksp$ undersaturation conditions

As the degree of saturation decreases a "critical pH" (Fosdick & Campaign, 1939) is reached, a level below which there is insufficient calcium and phosphate (plus fluoride for fluorapatite) to maintain the ion activity product at or above the solubility product. Near neutral pH values, salivary secretions are always supersaturated with respect to both apatites (Gron, 1973) providing a driving force for mineral deposition in enamel (hence the remineralising potential of saliva). Under conditions of supersaturation, spontaneous precipitation of calcium and phosphate is inhibited by the presence of salivary macromolecules like statherin (Hay, Schluckebier & Moreno, 1982; Mandel, 1985). These salivary inhibitors maintain conditions required for the integrity of the tooth structure while preventing the formation of

undersirable mineral deposits both in the salivary glands and on the surfaces of the teeth.

1.4 Fluoride and caries

The history of the discovery of the beneficial effect of fluoride on enamel, together with the major events that led to water fluoridation are shown in Table 1.1. Numerous clinical trials and large numbers of *in vitro* and *in vivo* studies have used different fluoride vehicles, study sites and protocols to demonstrate the anti-caries ability of fluoride. Many of these studies, however, give little information on the frequency and concentration of fluoride needed to give maximium protection against caries. Indeed most dosage regimens are entirely empirical. Selecting the correct fluoride concentration in a product is extremely important because fluoride is a potent cariostatic agent (Dean, 1942). In addition, fluorosis (enamel defects caused during pre-eruptive enamel maturation) in children, can be formed by inadvertently or intentionally swallowing fluoride during topical fluoride application (Ekstrand, 1987). Such problems regarding dosage regimens have arisen because of uncertainties in the mechanisms of fluoride action which are discussed in Section 1.5.

Initially it was thought that fluoride was only beneficial if it was administered systemically (ie. fluoride had to be ingested, absorbed and laid down in the forming

Table 1.1 Summary of the important findings in the history of water fluoridation

AUTHOR	DATE	FINDINGS
McKay	1901	First observation of "mottling" of enamel in Colorado Springs, USA.
Eager	1901	Independant observation of "mottling" of enamel attributed to some agent in the Naples water.
McKay & Black	1916	Epidemiological studies comfirmed Eager's suggestion that caries prevalence was less in areas where "mottling" of enamel was endemic.
Churchill	1931	Chemical analysis of water showed that "mottling" was due to high levels of fluoride, (up to 14 ppm).
Ainsworth	1933	First statistical data to show that caries experience in a fluoride area was lower than average.
Dean	1933 - 1936	The concentration of fluoride in water was correlated with the clinical severity of fluorosed enamel.
Dean	1945 - 1954	The addition of up to 1 ppm fluoride to water gave a caries reduction of 60 % with no significant fluorosis.

enamel). In 1938, Miller, using a rat caries-model, demonstrated that fluoride inhibited caries in erupted teeth. Klein (1946) and Backer-Dirks (1967) also showed that erupted teeth gained benefit from water fluoridation. This is the topical method of application and has come to play a major role in cariostasis. In 1942, Bibby carried out the first clinical trial using 0.1 % sodium fluoride for seven minutes, three times per year. Since then many different fluoride regimens have been employed and today topical fluorides are regarded as important caries preventive vehicles.

The original objectives in applying topical fluoride agents were (1) to provide protection where water fluoridation was not feasible; (2) to increase enamel fluoride concentration without the risk of fluorosis and, (3) to make fluoride available at the time of greatest caries risk. However, current knowledge suggests that the most important anticaries effect of topical fluorides is its presence in low, but sufficient concentrations, in an aqueous phase during a carious challenge (section 1.5.

Topical vehicles (fluoride compounds, aqueous solutions, gels, varnishes and dentifrices) fall broadly into two categories:

(1) professionally applied vehicles,

- and -

(2) self-applied vehicles.

Professionally applied vehicles are usually of a high

concentration and applied at infrequent intervals, for example, 2 % neutral sodium fluoride solution applied weekly at ages three, seven, ten and thirteen (Knutson & Armstrong, 1946; Knuston, 1948); 8 - 10 % stannous fluoride solutions (Mercer & Muller, 1961; Horowitz & Lucye, 1966); 1.23 % acidulated fluoride agents (Parmeijer, Brudevold & Hunt, 1963; Wellock & Brudevold, 1963; Wellock, Maitland & Brudevold, 1965) and 2 % fluoride varnishes (Heuser & Schmidt, 1968).

The recent revised understanding of the role of calcium fluoride [ie. the longer retention *in vivo* and the ability to convert to fluorapatite under cariogenic conditions (Ogaard, Rolla & Helgeland, 1983a, 1983b)] is the rationale for using high fluoride concentration in topical preparations.

Self-applied topical fluoride agents are for long-term use and can result in teeth receiving topical benefits both during and immediately after eruption. The most popular vehicle in this group is the dentifrice which contains as its main ingredient, either 0.2 % sodium fluoride (Torell & Ericsson, 1965) or 0.8 % sodium monofluorophosphate (Zipkin & McClure, 1951). Sodium monofluorophosphate was for some time the preferred formulation because of its greater compatibility with common dentifrice components particularly the abrasives. Recently, however, the use of new abrasive systems has made sodium fluoride-based formulations the

most popular.

The other main self-applied vehicle is the mouthrinse. Sodium fluoride mouthrinses are usually formulated at concentrations of either 0.2 % sodium fluoride (900 ppm F^-) for weekly use, or 0.05 % sodium fluoride (225 ppm F^-) for daily use (Torrel & Siberg, 1962; Torell & Ericsson, 1965; Koch, 1967; Rugg-Gunn, Hollway & Davies, 1973; Birkeland & Torell 1978; Ripa, Leske & Levinson, 1978). Mouthrinsing is not recommended for pre-school children because of their inability to control swallowing (Ericsson & Forsman, 1969).

The above mentioned fluoride vehicles have all contributed to the reported decline in caries. However, the physicochemical aspects of fluoride-enamel interactions (Section 1.5.3) shows that the most effective caries preventive fluoride regimens are frequent (daily) applications of fluoridated toothpaste and / or mouthrinsing.

1.5 Mechanisms of fluoride action

1.5.1 Introduction

Although the caries-inhibiting effect of fluoride has been studied for many decades, its mode of action is, however, still not well understood. It has been suggested that:

(1) fluoride incorporated into the enamel structure reduces its solubility in dilute acids;

(2) fluoride present in the aqueous phase around the tooth during a cariogenic challenge inhibits demineralisation and enhances remineralisation;

(3) fluoride inhibits the production of acids by microorganisms,

- and -

(4) fluoride affects tooth morphology.

1.5.2 Reduction of enamel solubility

This theory attempts to explain the cariostatic effect of fluoride by claiming that, when taken up in the apatite lattice (mainly during enamel mineralisation), this ion reduces enamel solubility in acids. The interaction of fluoride with the enamel crystallites is due to the unique chemical properties of this ion (i.e. high electronegativity, low dissociation energy, small radius, and the formation of strong, chemical bonds). These properties plus the fact that fluoride fits in the x-ion site with less strain makes fluorapatite less soluble in acids than hydroxyapatite.

Fluoride, present during the growth of enamel crystals, is able to eliminate impurities such as carbonates and magnesium. It also eliminates defects in the crystal lattice by filling in "voids" and also stabilizes the lattice by providing additional and stronger hydrogen bonds.

The use of fluoride for cariostatic purposes has until recently been based to a large extent on this theory.

Hence in the past, apart from systemic application, high fluoride concentrations were applied topically to increase fluoride concentration in the outer enamel layers. However several workers (Poulsen & Larson, 1975; Fejerskov, Thylstrup & Larsen, 1981; Sluiter & Purdell-Lewis, 1984; Weatherell, Robinson & Hallsworth, 1984) have indicated that fluoride incorporated in enamel does not significantly influence the resistance of the tooth to caries development. In addition, experiments by Nelson *et al.,* (1983) have shown that the dissolution rate of pressed synthetic hydroxyapatite was hardly influenced by the presence of 1000 ppm fluoride incorporated in the solid state. Today it is recognised that this explanation of fluoride action is an over-simplified concept because, relatively little fluorapatite exists in enamel, even that developed in fluoridated communities. Fluorapatite, with all its hydroxyl groups replaced by fluoride, contains 38,000 ppm fluoride whereas the fluoride content of enamel is usually only 500 - 1500 ppm fluoride (Weatherell *et al.,* 1977). Although the degree of substitution at the enamel surface may approach a level which causes a significant reduction in solubility, and could contribute to the cariostatic effect of fluoride, at best this is only a partial explanation of the anticaries mechanism of fluoride.

1.5.3 Fluoride in the aqueous phase

It is now well accepted that two major aspects of fluoride action are the inhibition of demineralisation,

including an effect on lesion formation (Larsen, 1973; Larsen & Fejerskov, 1977) and lesion histology (ten Cate & Duijsters, 1983a), and the enhancement of remineralisation. This theory claims that fluoride has to be present in the aqueous phase around the tooth, i.e. the saliva, the plaque / plaque fluid and the aqueous phase of the enamel pores, to affect a caries inhibition and to enhance remineralisation. The effect of fluoride in inhibiting or delaying demineralisation when present in the aqueous phase has been known for several decades (Manly & Harrington, 1959; Jeansonne & Feagin, 1974; Arends *et al.*, 1983; ten Cate & Duijsters, 1983a; Borsboom, van der Mei & Arends, 1985). Manly and Harrington (1959) also showed that the demineralisation rate of enamel exposed to a cariogenic solution containing 1 ppm fluoride was less than that of fluoride pre-treated enamel exposed to a fluoride-free buffer. Nonetheless, the fluoride acquired from the fluoride-rich buffer was only a fraction of the fluoride content of pre-incubated enamel. Several workers (Manly & Harrington, 1959; Speirs, Spinelli & Brudevold, 1963; Spinelli, Brudevold & Moreno, 1971) attributed the decreased acid solubility of enamel in fluoride-containing buffers to the formation of fluoride-rich mineral covering the enamel surface. The fluoride-rich mineral (which is thermodynamically the most stable) is formed, during a pH change, by transformation of the enamel mineral constituents involving a dissolution-precipitation mechanism and

requiring an intermediate aqueous phase. Therefore, acid-soluble mineral is first washed out and is displaced by an improved mineral. Ten Cate and Duijsters (1983a), suggested that bulk solution thermodynamic considerations, may not be relevant to explain the inhibitory effect of fluoride on enamel demineralisation. Their study showed that a rapid decrease in demineralisation was observed, not under conditions where fluorapatite became supersaturated, but when the solution became saturated with respect to calcium fluoride.

Some workers have shown experimentally that low fluoride concentrations stimulate fluorapatite precipitation (Brown, 1974; Amjad & Nancollas, 1979). Therefore, elevated fluoride levels in saliva will alter the balance between dissolution and crystal growth, since fluoride ions are available simultaneously with calcium and phosphate to diffuse into the lesion and precipitate as fluorapatite. This effect of fluoride in enhancing remineralisation has been demonstrated, both *in vitro* and *in vivo* (Koulourides, Cueto & Pigman, 1961; Koulourides *et al.*, 1974; ten Cate & Arends, 1977; Gelhard & Arends, 1984a; 1984b; Featherstone *et al.*, 1986).

1.5.4 Effect of fluoride on microorganisms

Although it is believed that fluoride has antimicrobial properties (Hamilton, 1977), there is no general agreement as to whether fluoride may significantly change the

microbial composition of plaque. The concentration of fluoride in plaque ranges from 13 - 55 mg F / g dry wt of plaque (Tatevossian, 1980). Plaque fluoride is present as either free, ionisable or as tightly bound with the percentage distribution of the three fractions changing as the plaque pH varies. It has been suggested (Hamilton, 1977) that all forms contribute to the overall inhibitory effect and that it is not always necessary for fluoride to be taken up by the bacterial cytoplasm in order to exert its effect (Edgar, Cockburn & Jenkins, 1981).

It is generally agreed that fluoride interacts with bacterial cells in a pH dependant way. Evidence from *in vitro* work suggests that acidification of the cytoplasmic compartment by dissociation of hydrofluoric acid is now recognised as a significant factor in the fluoride inhibition of growth and metabolism. For instance fluoride inhibits glycolysis at the enolase step by complexing with magnesium thus removing the latter ion from the catalytic site. This inhibition results in a reduction in the supply of phosphoenolpyruvate inhibiting sugar transport via phosphoenolpyruvate-phosphotransferase systems (Hamilton, Boyar & Bowden, 1985).

In the past clinical studies have claimed that topical fluoride may reduce plaque growth (Birkeland, 1972; Luoma, 1972). However, recent studies could not demonstrate similar findings. Indeed studies on *in vitro* growth and adaptation in the presence of fluoride, all

predict that oral bacteria will grow and survive in the levels of fluoride which are present in plaque under the natural conditions of fluoride ingestion.

1.5.5 Effect of fluoride on tooth morphology

Forrest (1956) and Cooper & Ludwig (1965) found that teeth formed in fluoridated areas are smaller and have shallower pits and fissures than teeth in non-fluoridated areas. There is no evidence, however, that altered tooth morphology can account for the anti-caries mechanism of fluoride.

1.6 De-/ remineralisation studies

1.6.1 Introduction

Several studies incorporating various models have been carried out to obtain information on the mechanisms of demineralisation and remineralisation and to study how factors such as fluoride concentration and duration affect remineralisation. These include *in vitro, in vivo* and *in situ* techniques. Many *in vivo* studies (von der Fehr, Loe & Theilade, 1970 ; Edgar *et al.,* 1978), are today either difficult to perform because the protocols involved are unlikely to receive ethical approval, or are restricted to the use of teeth designed to be extracted for orthodontic reasons (Holmen *et al.,* 1985a; 1985b; Ogaard, Rolla & Helgeland, 1983a, 1983b). The versatility of *in vitro* and *in situ* studies, pose no such problems and such methods are used frequently to give information on the

mechanism and efficacy of fluoride products.

1.6.2 Enamel source

Most experiments make use of bovine or extracted human enamel. Bovine enamel is frequently employed, primarily because it is more homogeneous and is easier to obtain (ten Cate & Arends, 1977; Feagin *et al.,* 1971; Borsboom, van der Mei & Arends, 1986). However, Featherstone and Mellberg (1981) compared the demineralisation of bovine, ovine and human enamel and found marked differences in their rates of demineralisation. In addition, Poole, Shellis and Tyler, (1981) found that the rate of lesion formation was greater in the enamel of non-human primates than in that of man, this difference in susceptibility being attributed to the lower porosity of human enamel. While synthetic hydroxyapatite (Langdon, Elliot & Fearnhead, 1980; Anderson & Elliot, 1985) has the advantage that it is a uniform substrate, experiments have shown that such substrates behave differently from intact enamel (Mellberg & Singer, 1977).

The method of preparing teeth for de-/ remineralisation work can also affect the enamel response to subsequent treatment. Inconsistent results can be caused by surface-related factors such as pellicle and the presence of high levels of fluoride. A number of workers (Pearce,1983; ten Bosch, van der Mei & Borsboom, 1984; ten Cate & Duijsters, 1982) therefore abraded the surface layer of the teeth. Others (Theuns *et al.,* 1983; Shellis,

1984; Kaufman *et al.,* 1984; Moreno & Zahradnik 1974) polished their specimens with pumice to ensure the pellicle was removed whereas Bergman and Lind (1966), Featherstone, Duncan and Cutress (1979), merely cleaned their teeth with water.

1.6.3 Bulk and thin sections of enamel

A variety of forms and shapes of enamel has been used for de- / remineralisation studies. These include whole teeth, powdered enamel (Leach, 1959; Brudevold *et al.,* 1963; Koulourides & Reed, 1964), slabs, cylinders and blocks of enamel (Mellberg, 1966; Mellberg *et al.,* 1985; ten Cate & Duijsters, 1982; Featherstone, 1983; Zimmermann *et al.,* 1985; Corpron *et al.,* 1986; ten Cate & Rempt, 1986; Arends & Dijkman, 1988), and thin sections of enamel (Featherstone & Silverstone 1982; Anderson & Elliot, 1985; Melberg, Castrovince & Rotsides, 1985; Wefel & Harless, 1985; Creanor *et al.,* 1986a; Strang *et al.,* 1987). Apart from the thin section technique, all the above studies employ a separate control specimen, or at best a different area of the same specimen. Enamel is not a homogeneous material and marked differences in mineral content and trace elements are present even in the same tooth (Robinson, Weatherell & Hallsworth, 1971). Hence, the use of a separate control specimen limits sensitivity in such studies. With thin sections of enamel the same enamel area can be studied longitudinally throughout an experiment. A section acts as its own control and thus enhances the accuracy of the method. For

this reason the "single-section" technique was the method employed in the work reported in this thesis.

1.6.4 Artificial lesion production systems

Early studies to produce artificial subsurface lesions made use of bacterial plaque grown *in vitro* on extracted teeth (Enright, Friesell & Trescher, 1932). However, it was soon realised that bacteria were not essential for the production of such lesions. Today, numerous different types of lesion-producing acid systems are employed, the most common using (i) gels, (ii) surface-preserving compounds (iii) buffers.

(i) **Gel systems:** These include the use of (a) gelatin (von Bartheld, 1961; Silverstone, 1967; Mellberg, 1980; Langdon, Elliot & Fearnhead, 1980; Kidd *et al.,* 1980; Creanor *et al.,* 1986a); (b) hydroxyethylcellulose gel (Gray & Francis, 1963; Groenveld, 1974; ten Cate & Arends, 1977); (c) methylcellulose gel (Gray & Francis, 1963). In these systems, a weighed amount of gel is used and acidified with lactic acid. In some systems, calcium and phosphate have been added (Kidd *et al.,* 1980; Creanor *et al.,* 1986a). The time required to produce subsurface lesions by these systems is long and variable, depending on the batch of gel used.

It has been suggested (Margolis, Murphy, & Moreno, 1985) that the gel medium with the organic and inorganic components acts as a substitute for plaque occurring *in*

vivo. This system is however difficult to define because of innate impurities (Pearce, 1983). In Chapter 5, a comparison of two lesion creation methods will be discussed.

(ii) **Surface-preserving systems:** Enamel is exposed, for a few days, to a demineralising medium of known composition containing a substance which decreases the dissolution rate of the mineral in the surface area of the tooth enamel. The surface preservers used include diphosphonates (Francis & Briner, 1973; Featherstone, Duncan & Cutress, 1979; Mobley, 1981; ten Cate, Shariati & Featherstone, 1985; Stookey *et al.*, 1985); natural macromolecules (Gray & Francis, 1963; Manson Hing *et al.*, 1972; Groenveld & Arends, 1975; Groenveld, Purdell-Lewis & Arends, 1975) and synthetic polymer gels (White, 1987).

Unfortunately, one potential problem with these compounds is their ability to poison crystal growth sites and by the same mechanism inhibit lesion remineralisation (ten Cate, Jongebloed & Arends, 1981). It has been suggested (ten Cate & Rempt, 1986) that the amount of diphosphonate used in demineralising buffers is only adsorbed on to the outer 10 - 20 µm of a lesion during the first stages of demineralisation so that this thin layer containing the surface preserver is similar to a protein layer preferentially adsorbed on to enamel (Moreno, Kresah & Hay, 1984). In addition, it has been shown (Arends & Dijkman, 1988) that *in vivo*,

diphosphonate- containing lesions remineralise in a way similar to *in vivo* lesions without diphosphonate.

(iii) **Buffer systems:** These systems make use of chemically well-defined buffer solutions containing organic acids plus calcium and phosphate ions. Several workers (Besic, 1953; Coolidge, Besic & Jacobs, 1955; Moreno & Zahradnik, 1974) showed that the presence of calcium and phosphate was required to obtain subsurface lesions. Acetate buffers of varying hydrogen ion concentrations are commonly used, together with different amounts of calcium and phosphate, thus making it possible to work with varying degrees of saturation with respect to hydroxyapatite. For a solution to demineralise enamel, and to give subsurface demineralisation, it is generally agreed that the solution should be undersaturated with respect to hydroxyapatite and supersaturated with respect to fluorapatite (Larsen, 1974). However, subsurface lesions have been prepared (Theuns, Driessens & van Dijk, 1986) with a solution undersaturated with respect to both apatites. Furthermore, work on synthetic apatites (Anderson & Elliott, 1987) showed that subsurface lesions could be formed in the absence of fluoride and without added calcium and phosphate in the demineralising medium.

1.6.5 In vitro studies of remineralisation

Early *in vitro* experiments to study remineralisation have, in the past, been carried out on early carious enamel

(Muhlemann, 1964; Silverstone & Poole, 1968). Conditions which affect the rehardening of surface softened enamel have, been well documented (Pigman, Cueto & Baugh, 1964; Feagin *et al.*, 1971). Variables such as pH, ionic strength, calcium, phosphate and fluoride concentration in the mineralising solution were studied for their effects on initial rates of enamel remineralistion.

However, over the past decade, remineralisation studies have concentrated on subsurface lesions (ten Cate & Arends, 1977; Groeneveld, Theuns & Kalter, 1978; Clarkston, Wefel & Feagin, 1986) *In vitro* remineralisation is achieved by immersing specimens in a solution containing calcium and phosphate, in amounts comparable to those found in natural saliva (ie. the ion activity product with respect to hydroxyapatite in the solution should be greater than the solubility product with respect to hydroxyapatite). In addition the presence of fluoride has been shown to enhance this remineralising process (Koulourides, Cueto & Pigman, 1961; Feagin *et al.*, 1971).

In vitro remineralising solutions are difficult to prepare because such systems are metastable and soon precipitate, thus becoming ineffective. To increase the stability and range of pH over which the remineralising solution is active, many workers (Koulourides, Feagin & Pigman, 1968; ten cate & Arends, 1977; Stookey *et al;* 1985) have added sodium chloride. Others (Featherstone *et al.,* 1983) have added ions like tartrate to partially

complex metal ions and promote their transport into carious lesions. Remineralisation can be achieved, either throughout the lesion, or can be restricted to the surface of the lesion, giving lesion "arrestment" depending on the concentrations of calcium, phosphate and fluoride (Silverstone *et al.,* 1981).

Shellis and Marshall (1987) studied crystal growth in remineralising solutions used by several workers (Pigman, Cueto & Baugh, 1964; Silverstone *et al.,* 1981; Featherstone *et al.,* 1983) and found such solutions to be unstable and not very successful in remineralising early carious lesions as had been reported previously.

The technique of pH cycling is frequently used to simulate intra-oral pH fluctuations, so that preventive regimens incorporating fluoride can be tested. With this technique, enamel specimens are usually exposed to one extended demineralisation cycle and one remineralisation cycle per day, for a period of three or four weeks. By altering the pH of the medium, the efficiency of fluoride incorporation into enamel is increased (Mallaowalla & Myers, 1961; Brudevold *et al.,* 1963; Ramsey *et al.,* 1973; Duff, 1976), and information on the mechanism of de- and remineralisation (ten Cate & Duijsters, 1982; Buskes, Christoffersen & Arends, 1985; Featherstone *et al.,* 1986; Gerrard & Winter, 1986; ten Cate & Simmons, 1986; White, 1987; ten Cate *et al.,* 1988; Damato, Strang & Stephen, 1988) has been obtained.

The major disadvantages of pH cycling are that the method is labour-intensive and does not represent demineralisation by a series of Stephan-like curves. However these problems have recently been overcome because pH cycling has been automated (ten Cate & Simons, 1989; Page, 1989).

In vitro work has many advantages over *in vivo* / *in situ* techniques. These include: (1) greater control over conditions as the effect of a single parameter eg. fluoride concentration, can be studied without the interference of such factors as diet, oral hygiene, fluoride levels of saliva and plaque; (2) large numbers of specimens can be easily dealt with; (3) the uncertain co-operation of volunteers is not required and; (4) the technique is relatively simple and inexpensive.

Thus the above review shows that *in vitro* methods provide a simple experimental approach to demonstrate de- / remineralisation of enamel and could be used to obtain information on fluoride preventive regimens.

1.6.6 In vivo / in situ studies

Several *in vivo* studies (von der Fehr, 1965; Backer-Dirks, 1966) have shown that early carious lesions remineralise *in vivo*, especially with good oral hygiene and fluoride treatments. *In vivo* studies, excluding those using teeth due for orthodontic extraction (Ogaard, Rolla & Helgeland, 1983a; 1983b; Holmen *et al.*, 1985a, 1985b), give little

information regarding remineralisation mechanisms. The use of *in situ* models, in which artificial or natural enamel defects on extracted teeth are positioned in partial or total protheses, combine some of the advantages of *in vivo* and *in vitro* methods. They provide a natural environment in which remineralisation may occur. In addition, changes in mineral content can be assessed by microradiography of the removable enamel specimens.

Koulourides and Volker (1964) introduced an *in situ* model to measure microhardness changes in the caries-like lesions formed on tooth slabs. In 1974, Koulourides and co-workers developed the ICT (intraoral cariogenicity testing) model for studies of fluoride incorporation into bovine enamel. Since then several *in situ* models have been described. In such studies plaque is allowed to develop around the specimens and many models encourage such plaque accumulation by using a Dacron gauze. In most experiments, enrolled volunteers wear a partial or full denture that is sufficiently large to allow the placement of one or two enamel slabs. In some studies (Featherstone *et al.*, 1982; Corpron *et al.*, 1986; Creanor *et al.*, 1986a; Hellwig, Klimek & Wagner, 1987) non-denture wearers are recruited and these are fitted with removable acrylic appliances.

In situ studies have demonstrated that, under intraoral conditions, remineralisation takes place and is enhanced when a fluoride agent is used. Most *in situ* studies have

been designed to measure fluoride uptake and its incorporation into enamel (Koulourides *et al.*, 1974; Mobley, 1981; Mellberg & Chomicki, 1983; Stookey *et al.*, 1985; Zimmermann *et al.*, 1985; Corpron *et al.*, 1986; ten Cate & Rempt, 1986; Clark *et al.*, 1988). Several investigations including many of those cited above, use additional techniques to demonstrate changes in mineral content of specimens.

In recent years *in situ* models have been employed to compare different fluoride products and to study the dose-response relationship between fluoride concentration and *in situ* remineralisation of enamel specimens (de Kloet, *et al.*, 1986; Goorhuis & Purdell-Lewis, 1986; ten Cate & Rempt, 1986; Creanor *et al.*, 1987; Schafer, 1989). In Chapter 8, an *in situ* study, which makes use of thin sections of enamel, is described to show a fluoride dose-response with sodium monofluorophosphate dentifrices.

The *in situ* model (Creanor *et al.*, 1986a; 1986b) has also been used extensively by Macpherson (1988) to study the microbiology associated with *in situ* de-/remineralisation.

1.7 Assessing mineral content changes

Changes in enamel mineral content have been measured by various techniques, the most commonly used include: (1) microradiography (Angmar, Carlstrom & Glas, 1963; Groenveld, 1974; Arends & Gelhard, 1983; Gelhard &

Arends, 1984b; Bergstrom, Fox & Higuchi, 1984; Josselin de Jong & ten Bosch, 1985; Ogaard *et al.,* 1986; Strang *et al.,* 1987); (2) polarising microscopy (Darling, 1956; Silverstone, 1968); (3) microhardness (Arends, Schuthof & Jongebloed, 1980; Featherstone *et al.,* 1983) and (4) chemical measurements (ten Cate & Duijsters, 1982; 1983a, Borsboom, van der Mei & Arends, 1985).

1.7.1 Microradiography / microdensitometry

Microradiography is the application of soft X-rays to study the degree of mineralisation and demineralisation of a tissue. Thin sections (approximately 100 μm) from teeth are placed in contact with a glass slide bearing a fine-grained emulsion capable of high resolution and these are secured in holders. The holders are exposed to monochromatic X-rays produced at low voltage from a diffraction tube with a copper target and a nickel filter.

A 1:1 size X-ray absorption image is produced and this can be examined microscopically. Microdensitometry is used to measure the optical density of the image from which the mineral content can be determined (Angmar, Carlstrom & Glas, 1963). Microradiography / microdensitometry is a rapid, non-destructive and direct method since it measures the actual amount of mineral in a specimen. It also permits the accurate determination of the mineral content in very small volumes of enamel (Angmar, Carlstrom & Glas, 1963). This is the method used in the studies reported in this thesis and will be discussed in Chapter 2.

1.7.2 Polarising microscopy

Polarising microscopy is used to show changes in enamel porosity. It is a simple (when used qualitatively), non-destructive method for studying mineral distribution in carious enamel. This procedure makes use of the birefringence of the mineral component of enamel. Enamel is said to have a negative intrinsic birefringence due to its orientated crystal component and a positive form birefringence due to the presence of small orientated pores.

When thin, ground sections are examined in polarised light, the section is placed in an imbibition medium (eg. water, quinoline or naphthalene). The magnitude of form birefringence is dependant on the refractive index of the solid and that of the medium filling the pores, as well as on the volume fraction occupied by the solid. Thus, when carious enamel is examined after imbibition in water, the form birefringence will be produced if the spaces created in the tissue are large enough to admit water. The observed birefringence, which is the sum of the intrinsic birefringence and the form birefringence, can be measured using a suitable optical compensator in conjunction with a polarising microscope. Polarising microscopy has been applied frequently (Silverstone, 1966; 1967; 1968; Kidd, 1983) as it gives much ultrastructural detail. This technique is, however, only considered to be semi-quantitative. It has been shown (Shellis & Poole 1985) that estimation of pore volume from form

birefringence, is not reliable. This method of estimating enamel mineral content is, therefore, only possible when it is used in conjunction with other calibrating techniques, especially microradiography.

1.7.3 Microhardness

The principle of the microhardness technique involves the measurement of the penetration of a Knoop diamond under a fixed load into enamel. On a non-elastic material, the diamond indents the surface, the maximium length of which defines the penetration depth. The microhardness test was initially developed to assess the hardness of homogeneous materials. However, it was later introduced for experimental caries studies (Caldwell *et al.,* 1958; Newbrun, Timberlake & Pigman, 1959), the assumption being that the measured hardness is related to the degree of porosity of the superficial enamel layers. This technique is both time-consuming and destructive, and precautions must be taken to shelter the microhardness tester from vibration. The indenter must descend in a perpendicular direction on a horizontal plane and the test surface must remain intact as seen by microscopic examination (x 200 - 500 magnification). This test is usually carried out on normal bulk enamel (Koulourides *et al.,* 1974; Gelhard, ten Cate & Arends, 1979, Arends, Schulthof & Jongebloed, 1979, 1980), or on polished cut surfaces (ten Cate, Shariati & Featherstone, 1985). Featherstone and workers (1983) have shown that when using polished cut surfaces the square root of the Knoop Hardness Number (KHN)

calculated from the indentation length is linearly proportional to the volume per cent mineral determined by microradiography.

There are several disadvantages associated with this test namely: (i) a polished surface is required; (ii) the validity of the test depends on the assumption that the change in mineral density is similar at all points of the test surface, and (3) because the method is destructive, a separate control specimen is required.

1.7.4 Chemical techniques

Chemical techniques are among the indirect methods for measuring mineral content changes in enamel. The methods frequently used include the measurement of calcium and / or phosphate concentration changes from solutions in which bulk enamel specimens have been placed under specific conditions. A large lesion surface and a small volume of solution is used so that concentration changes in the solutions can be accurately detected. Atomic absorption spectroscopy is the simplest and most efficient way of measuring calcium changes in small samples. Phosphate is generally measured colorimetrically (Chen *et al.,* 1956). A good correlation has been found between the calculated rate of demineralisation from microradiography and chemical analysis (ten Cate & Duijsters, 1983b; Theuns *et al.,* 1985). However, the rate of demineralisation obtained from chemical analysis is found to have smaller standard deviations than that obtained from microradiogrphy since

local variations in the enamel specimen would have little influence on the chemical results. This method like most other techniques requires a separate control specimen.

Chemical analysis involving microsampling and microanalytical techniques have been employed to obtain information on the variation and composition of enamel mineral (Robinson & Weatherell, 1968; Robinson, Weatherell & Hallsworth, 1971).

1.8 Aims

As mentioned in Section 1.4 optimal fluoride regimens in terms of concentration, frequency of application and duration are still empirically based. Recent evidence on the mode of action of fluoride indicates that the most effective caries preventive treatments should be based on frequent applications of low fluoride concentrations (Featherstone & ten Cate, 1988).

Accordingly, the main aim of this thesis was to develop a pH cycling model to investigate the effect of fluoride on de-/ remineralisation processes. The development of the model included:

(a) a demineralisation study to test the advantages of using thin sections of enamel.

(b) selection of pH and other experimental conditions such that mineral content changes in the enamel specimens could be quantified.

(c) choosing a lesion preparation method which would produce standardized subsurface lesions capable of

responding strongly to various experimental protocols.

This pH cycling model was used to demonstrate *in vitro* (i) the effect of continuous low fluoride levels on enamel de-/ remineralisation, and (ii) to show that an optimal fluoride concentration for maximium caries protection exists.

The aim of the final part of the project was to improve the *in situ* model described by Creanor *et al.* (1987) to obtain information on *in situ* de-/ remineralisation processes and to see if it was possible to demonstrate a fluoride dose-relationship with sodium monofluorophosphate dentifrices.

Chapter 2

Materials and methods

2.1 Introduction

This chapter describes the principal techniques used in the studies reported in later chapters. These include the preparation of single sections of human dental enamel, the preparation of artificial carious lesions and the quantification of enamel mineral content. Fluoride measurement techniques are also described. The materials used throughout the course of this study are detailed in Appendix I.

2.2 Preparation of enamel specimens

2.2.1 Tooth supply

The tooth enamel was obtained from human premolar teeth previously extracted for orthodontic reasons, and obtained from various practices in the Glasgow area (water fluoride content = 0.02 ppm).

2.2.2 Tooth preparation

In this study, teeth were collected in screw-cap containers containing 0.1 % thymol solution. Prior to lesion creation, teeth were first washed in warm soapy water, in batches of twenty and cleaned with a pumice / alcohol mixture to remove any pellicle and debris.

Pumicing was performed manually and was not intended to remove any significant amount of outer enamel. After rinsing in cold running water for a few minutes, the specimens were air-dried. A length of adhesive tape 400μm in width, was wrapped round each tooth four times, leaving a gap of about 300 μm between each strip. A thick layer of acid-resistant nail varnish was then applied to the buccal surface of each tooth and left to dry. The adhesive tape was then removed, leaving four exposed areas across the buccal surface. The rest of the tooth, including the root, was varnished and left to dry overnight. Each tooth was examined, using a stereomicroscope with x 10 magnification, to ensure exposed areas were free of any adhesive or nail polish, prior to immersion in a demineralising medium.

2.2.3 Preparation of subsurface lesions

Two artificial caries systems were used to create subsurface lesions on whole teeth. Initially the gelatin system (Silverstone, 1967; Kidd *et al.*, 1980) was employed, but, as the problems associated with this system (Section 1.6.4) became apparent, a standardized chemical system was used in later studies.

(1) **Gelatin system.** Litre batches of ten per cent gelatin were prepared at 37° C, at which temperature the gelatin exists in a sol state. One millimolar (mM) calcium triphosphate was added to the gelatin preparation, in addition to a few crystals of thymol, which prevented

bacterial growth. The gelatin was acidified to pH 4 with 80mM lactic acid and stored in a glass bottle at room temperature. Prior to use, the gelatin medium was placed in a water bath at 37 °C to bring it to the sol state, after which 10 mL aliquots were placed in glass vials. Teeth were suspended in the gel in separate universal bottles and left undisturbed at room temperature for periods which varied from 10 to 12 weeks.

Information about impurities present in the gelatin was obtained from the suppliers. These included (i) arsenic (1 ppm), (ii) lead (5 ppm), (iii) copper (50 ppm), (iv) zinc (100 ppm) and (v) sulphur dioxide (1000 ppm). The fluoride content of the gelatin medium was measured using a specific fluoride electrode and found to be 0.15 ppm.

(2) **Buffer system.** The demineralising solution pH 4.5, ion product activity (pI of 126) was prepared regularly in one or two litre batches. It contained 3.1 mM calcium chloride, 3.1 mM sodium dihydrogen orthophosphate and 50 mM (2.875 mL) glacial acetic acid. Double-distilled deionised water was added and the pH brought up to 4.5 with 1 M sodium hydroxide. The solution was transferred to a one litre volumetric flask and made up to the mark with double-distilled deionised water. Batches of five teeth, each with four exposed windows, were placed in 50 mL of the demineralising solution. Two days later, this solution was changed and demineralisation continued for a further

three days. The fluoride content of the solution was measured regularly and consistently found to be less than 0.02 ppm. Artificial caries on the buccal surface of a premolar tooth prepared using this method are shown in Figure 2.1.

2.2.4 Section preparation

Enamel sections were prepared from teeth which had been exposed to one of the demineralising systems described in Section 2.2.3. Once the demineralising period was over, the teeth were washed several times with acetone to remove the nail varnish. Each tooth crown was then halved mesiodistally using a dental drill, thus freeing the buccal surface containing the artificial carious lesions. This portion of tooth was mounted on to an acrylic block using cyanoacrylate and the adhesive allowed to dry overnight.

Enamel is hard and brittle and therefore difficult to cut without shattering (Bovis, 1968). The method used was standardized throughout, sections being cut to a thickness of approximately 250 µm using a circular diamond saw microtome (E. Leitz Instrumental Ltd; Luton, England), operated at a slow speed and cooled by running water. In this way, a maximium of four high quality sections were obtained from each tooth.

Figure 2.1 Four areas of artificial enamel caries on the buccal surface of a human premolar tooth

2.2.5 Planoparallel sections

Planoparallel sections for the quantification of mineral content were prepared by hand-grinding the sections on a glass plate using aluminium oxide or silicon carbide as abrasives and a brass weight. The final thickness of the sections was measured using a micrometer(Mitutoyo, Tokyo, Japan) to an accuracy of 1 µm. Four or five measurements of the section thickness were taken from the incisal to the cervical ends and the mean value was calculated. When the measured thickness along the tooth varied by more than 5 µm, specific values corresponding to the lesion position were used in subsequent analyses. The final thickness of sections used in this study varied from 100 µm to 145 µm.

2.2.6 Varnishing sections

Before exposing the sections to any experimental protocol, all aspects of the specimens, apart for the outer enamel containing the artificial lesions, were varnished with acid-resistant nail polish. Varnishing was carried out at x 10 magnification using a stereomicroscope. Special care was taken to ensure the varnish covered all cut aspects, so that these parts of the specimen were totally isolated from the experimental medium.

2.3 Solutions for pH-cycling studies

The demineralising solution used as an acid challenge was prepared in the same way as the buffer solution described

in Section 2.2.3 except that it contained 2 mM (0.2219 g) calcium chloride, 2 mM (0.3120 g) sodium dihydrogen orthophosphate and 50 mM glacial acetic acid. The pH was adjusted to 4.8 with 1 M sodium hydroxide.

The remineralising solution, or artifical saliva (2 mM calcium chloride, 2 mM sodium dihydrogen orthophosphate; pH 6.85) was stable for about 24 hours after which a precipitate was generally evident. To account for the metastability of this solution, two stock solutions were prepared, one containing 4 mM calcium chloride and 4 mM sodium dihydrogen orthophosphate and the other containing 2 mM sodium hydroxide. Prior to use, equal volumes of the two solutions were mixed and the pH checked to ensure it was within the expected range.

2.4 Fluoride analysis

2.4.1 Introduction

Previous methods for determining fluoride in enamel were tedious, involving a diffusion procedure to separate the fluoride with subsequent estimation by a colourimetric procedure (Wharton, 1962). Introduction of the fluoride ion activity electrode has permitted direct measurement of the free fluoride ion concentration, requiring no separations and no adjustment of sample size. The fluoride electrode (model 94-09, Orion Research Incorporated Ltd., Massachusetts, USA) consists of a sensing element which, when placed in a solution

containing fluoride, sets up an electrode potential dependant on the level of free fluoride ion in solution. This potential is measured against a constant reference potential with a specific ion meter. The electrode response is relatively slow at low fluoride concentrations (< 0.1 ppm). However Ekstrand (1977), showed that by using certain precautions, direct measurement of such low fluoride concentrations is possible. The fluoride electrode is used widely in dental research, particularly because the standard techniques can be modified to suit the particular requirement of individual studies (Birkeland, 1970; Venkateswarlu, 1975; Hallsworth, Weatherell & Deutsch, 1976; Vogel, Chow & Brown, 1983; Retief *et al.*, 1985; Vogel & Ekstrand, 1989; Tyler & Comer, 1985; Tyler & Poole, 1989).

2.4.2 Fluoride measurements

In this thesis the direct and indirect calibration methods were utilised for determining fluoride concentration in samples. Both methods required the use of a total ionic strength adjustor (TISAB II or III) to maintain a sample pH of 5 - 5.5 and prevent hydroxide interference or formation of hydrogen complexes of fluoride. Fluoride attacks glass, therefore disposable plastic-ware, which also prevented contamination, was used.

The direct method of analysis (Orion Instruction Manual)

was employed to determine fluoride levels above 0.4 ppm and also for comparison with the indirect method. For salivary and plaque fluoride measurements (Chapter 8) where fluoride values lie in the nonlinear portion of the calibration curve, the indirect method was applied. Using semilogarithmic graph paper, a calibration curve was prepared, every three hours, by plotting the relative millivolt values on the linear axis and the standard concentration values on the logarithmic axis. A total of eight fluoride standard solutions prepared using double-distilled deionised water (F < 0.005 ppm) and containing <0.005, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1 and 0.5 ppm fluoride, were used.

2.5 Microradiography and microdensitometry

2.5.1 Introduction

Quantitative contact microradiography is one of the most sensitive and non-destructive methods available for determination of enamel mineral content (Angmar, Carlstrom & Glas, 1963). This technique is widely used to study the loss or gain of mineral from enamel sections (Groenveld, Theuns & Kalter, 1978; ten Cate & Duijsters, 1983b; Theuns *et al.*, 1983; 1984a, 1984b; Mallon & Mellberg, 1985; Strang *et al.*, 1987; Theuns, Driessens & van Dijk, 1986) and was the method of analysis available for work reported in this thesis.

Certain conditions are essential for the accurate

quantification of mineral content using micro-radiographic techniques. First of all, monochromatic radiation must be used; this is achieved using a nickel filter to absorb all unwanted rays from the polychromatic source. Secondly, the sections need to be planoparallel. Thirdly, a reference system is necessary for calibrating the absorption of incident X-rays; this consists of an aluminium wedge containing six steps placed along one length of the plate. Finally, the beam of X-rays needs to be homogeneous and of sufficient width. In this study, the strength of the beam varied by about 13 % along one axis and by 1 % along the other axis. Factors taken into consideration when selecting the exposure time and tube voltage, included the type of photographic film and the working distance.

2.5.2 Microradiography of enamel sections

Microradiographs of enamel sections were taken at regular intervals depending on the experimental protocols. Sections were first washed in acetone and amyl alcohol, to remove the varnish, then successively placed in ethyl alcohol, methyl alcohol and water, each for approximately one minute. After removing excess water from the surface of the sections, these were mounted on Kodak High Resolution Plates (Type 1A) together with the aluminium wedge. The plates, secured in holders, were exposed for 20 minutes to a Cu ($K\alpha$) X-ray source (Marconi TX 12) in an Enraf Nonius generator operating at 20 kv and 30 mA, at a focus-source distance of 300 mm. They were then

developed according to the manufacturer's instructions. A microradiograph of sections together with an aluminium stepwedge is shown in Figure 2.2.

2.5.3 Microdensitometry

The microdensitometric apparatus was based on a Leitz ASBA Image Analyser (Fig. 2.3). This unit consisted of a microscope fitted with a black and white video camera and a computerised image analyser which digitised the video signals from the camera.

Firstly, the grey level value (ie. optical density) for each thickness of aluminium was recorded and a fourth order polynominal calibration curve fitted to the data. A microradiograph of the enamel section containing artificial lesions was then positioned on the microscope stage and images of each artificial lesion (Fig. 2.4) digitised into 256x256 pixels (1 pixel = 3 µm). The digitised data were then transferred to a BBC microcomputer (Acorn, Cambridge, England) for analyses.

The image of the lesion was displayed on the computer monitor and an area of interest within the lesion was delineated (Fig. 2.5). The average microdensitometric profile within the area was calculated in terms of grey levels (optical density). These measurements were then converted to their % volume mineral content, using the equation derived by Angmar, Carlstrom and Glas (1963), The data were stored on a floppy disc for subsequent analyses and a hard copy of the displayed lesion, with the

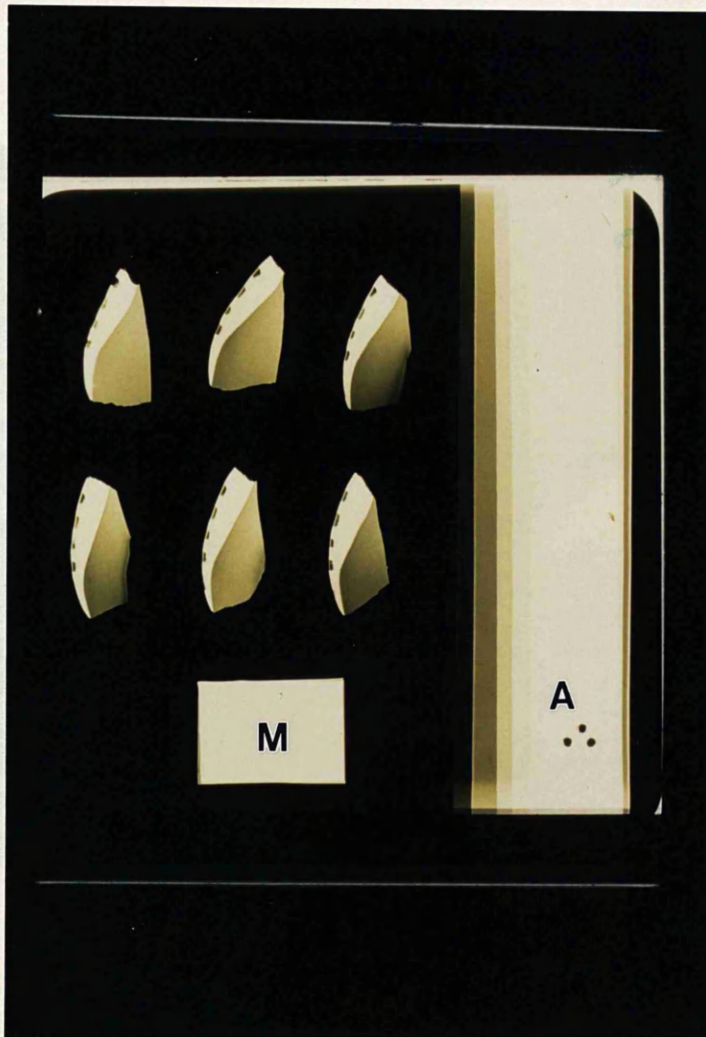


Figure 2.2 Typical microradiograph of six sections, together with an aluminium stepwedge (A) and marker (M) (A separate stepwedge calibration was used for every lesion on all six sections).

Figure 2.3 The T.V. video-camera and microscope (a), the Leitz ASBA image analyser (b), and the BBC-B microcomputer (c) used for microdensitometry

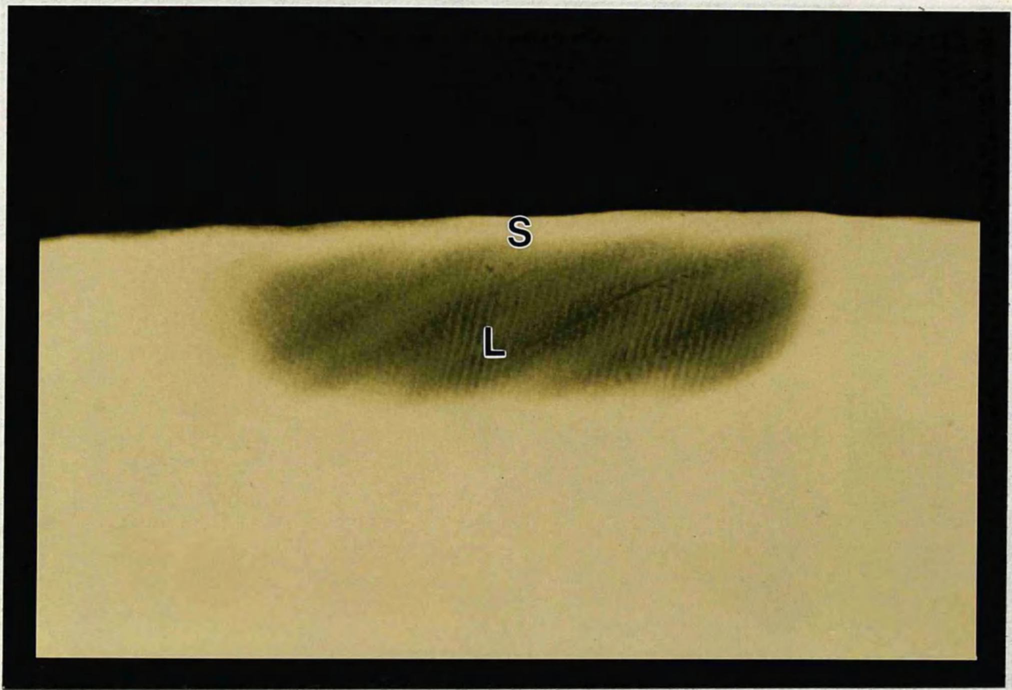


Figure 2.4 Microradiograph of a longitudinal ground section with an artificial subsurface carious lesion showing the surface layer (S) and lesion body (L)

the outlined "area of interest," was obtained using a "frame grabber" ((Video Graphic Printer UP-701 Sony, Japan). This picture ensured the same area of the lesion was measured on different occasions.

2.5.4 Microdensitometric analysis

Microdensitometric profiles of the lesions under investigation were analysed using software written in house by the Caries Research Group in Glasgow. Figure 2.6 is a schematic representation of a lesion profile. The maximum % volume mineral of sound enamel was arbitrarily taken as 80 % and all data normalized accordingly. In previous studies, the measured % volume mineral content of sound enamel varied from 82 % to 87.2 % (Angmar, Carlstrom & Glas, 1963; Bergmann & Lind, 1966; Groenveld, 1974; Hoppenbrouwers, Driessens & Borggreven, 1986).

The parameters used for the quantification of mineral are shown in Figure 2.6. These included: (i) Δz; (ii) % volume mineral content of the surface zone (SZ), and (iii) the % volume mineral content of the lesion body (LB).

(1) Δz is a measure of the total mineral lost from the lesion. It is denoted by the shaded area in Figure 2.6. This parameter was calculated from the 20 % volume mineral on the initial slope of the microdensitometric profile, to a point "S" on the sound enamel. Many different definitions of Δz appear in the

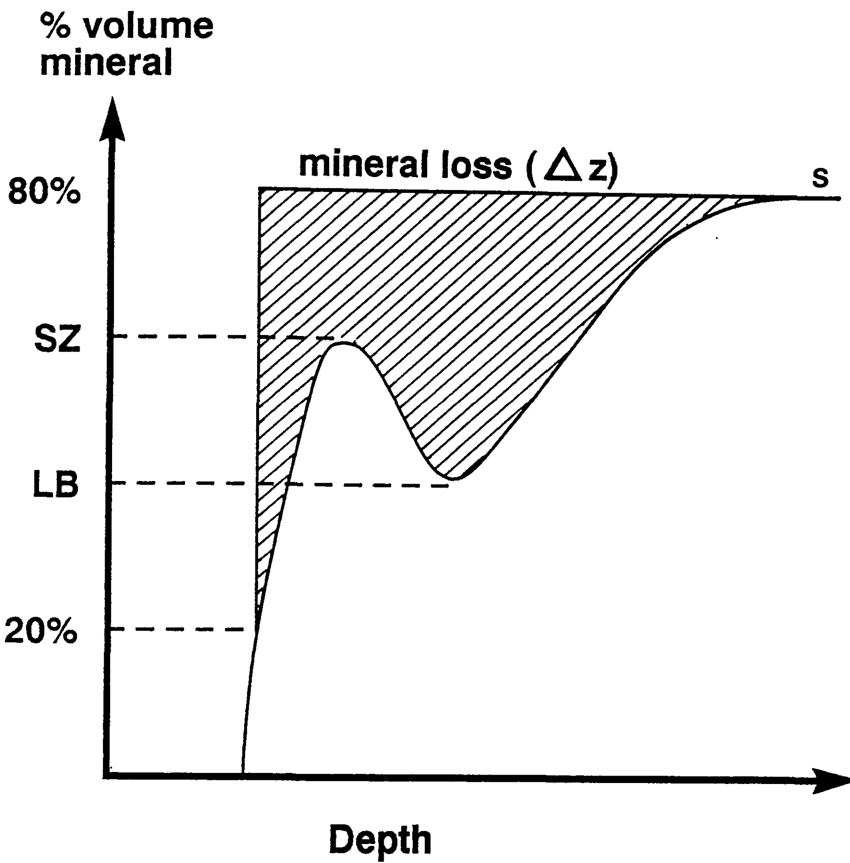


Figure 2.6 Schematic representation of a lesion profile illustrating the % volume mineral of the lesion with depth.
Δz is the total mineral loss represented by the shaded area.
(SZ) is the % volume mineral of the surface point.
(LB) is the % volume mineral of the lesion body.

literature, some starting at 0 % on the initial slope (Arends & ten Bosch, 1986; Dijkman, Schuthof & Arends, 1986) and others at the surface zone maximium (Arends & Gelhard, 1983; Mallon & Mellberg, 1985; De Brynn *et al.*, 1988; Leach, Lee & Edgar, 1989). In this project, the calculation of Δz commenced at 20 % because it was found that values close to zero gave high errors due to flattening out of the calibration curve.

Initially, when analysing the mineral content of the baseline lesion, point "S" was selected visually by the operator. In subsequent analyses of the same lesion point "S" was determined automatically by the computer, so that the distance between the 20 % volume mineral point and point "S" was constant. When lesions extended beyond the original point "S, then the baseline lesion was re-analysed selecting a new point "S" further away from the lesion. Subsequent lesions were also re-analysed using this point "S". Since Δz is a measure of the amount of mineral lost from a lesion, the bigger the value of Δz the greater the loss of mineral. Recently it has been shown (Strang *et al.*, 1987) that the rate of de-/ remineralistion of a lesion is affected by its initial Δz value. Therefore, in work carried out after this finding, only artificial lesions with an initial Δz values between 2000 - 4000 % volume mineral x µm (subsequently referred to as % vol. min. x µm) were analysed.

(2) The volume percent mineral content (subsequently referred to as % vol. min.) of the surface zone (SZ) is taken as the volume % mineral content of the maximium point in the microdensitometric profile (Fig. 2.6) with high values indicating a well mineralised surface layer.

(3) The lesion body value (LB) is the volume % mineral content (subsequently referred to as % vol. min.) of the minimium point in Figure 2.6. Substantial amounts of mineral are lost or gained from this region.

At the end of a demineralising or remineralising experiment, the rate of de-/ remineralisation was calculated by further analyses of the microdensitometric parameters. For each lesion, the values of each parameter were plotted against time. An example of a plot of the successive Δz values from one lesion are shown in Figure 2.7. A straight line was drawn from the data using a least squares technique and its slope taken as a measure of the de-/ remineralisation rate for that lesion and for that parameter. The standard errors (SE) of individual lesions were derived from the computed variance for the slope in the regression analysis (the standard error of the mean being used with the mean values reported throughout this thesis).

The negative of the slope was used for the Δz parameter so that for all parameters (ie. Δz, SZ and LB) positive values of the mineralisation rate indicated remineralisation.

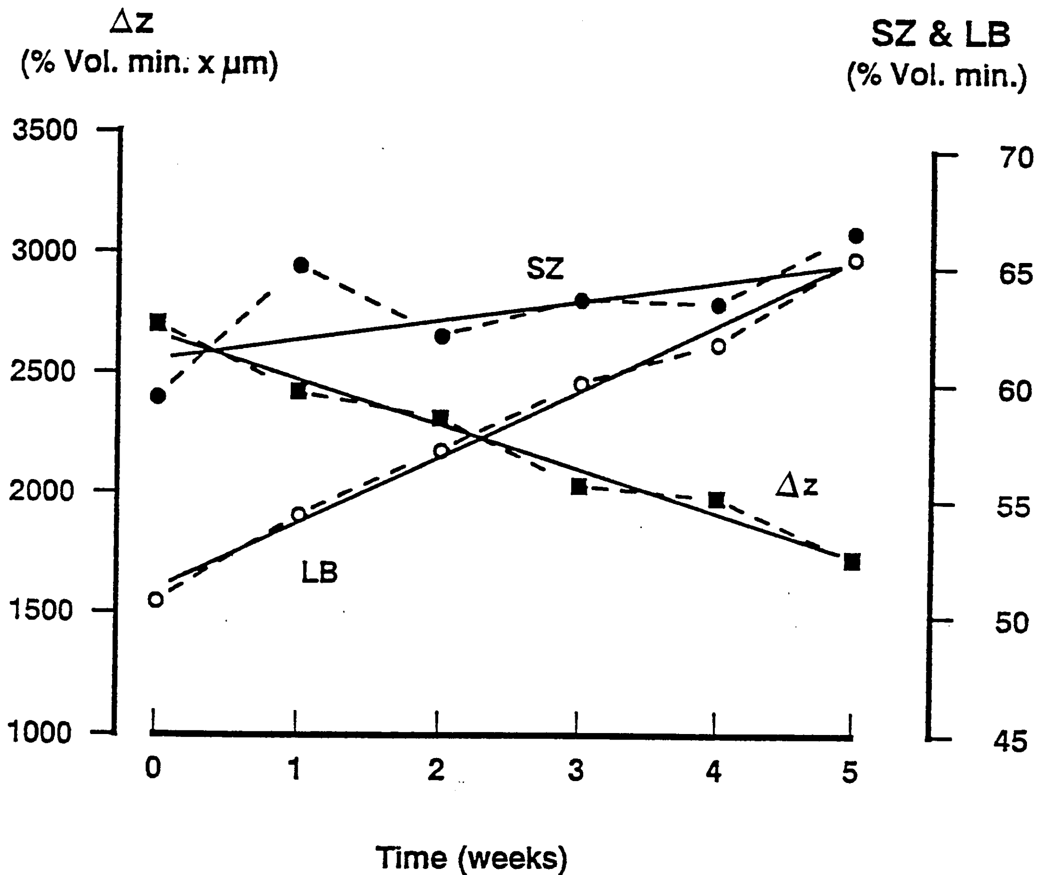


Figure 2.7 Plots of Δz, surface zone (SZ) and lesion body (LB) mineral contents, against time for a lesion.
Solid lines represent the least squares regression lines.
(A remineralisation experiment)

Chapter 3

In vitro demineralisation of enamel sections and slabs

3.1 Introduction

One of the problems associated with enamel de-/remineralisation studies is the variation in susceptibility of the enamel tissue to cariogenic challenge. Most workers have tried to overcome this problem by using a control specimen from the same tooth. However, since variation exists even within the same tooth (de Groot, Borggreven & Driessens, 1986), the use of a separate control tissue limits the sensitivity of such studies. In addition, in studies using bulk enamel specimens, the mineral content can usually be determined at the end of the experiment. As discussed in Section 1.6.3 the "single-section" technique, using thin sections of enamel, excludes biological variation in longitudinal studies because it enables the same area of enamel to be examined repeatedly throughout an experiment. The area of enamel under investigation can, therefore, be used as its own control. Hence this method is undoubtedly superior to the use of whole teeth or slabs of enamel.

The "single-section" technique was first used in caries research by von Bartheld (1980). Since then, several workers have used sections of enamel for de-/remineralisation studies, both *in vitro* (Featherstone &

Silverstone, 1982; Wefel & Harless, 1985; Kidd *et al.*, 1980) and *in situ* (Creanor *et al.*, 1986a; Strang *et al.*, 1987; Wefel, Maharry & Jensen, 1987; Mellberg, Castrovince & Rotsides, 1986; Mellberg *et al.*, 1988).

In a recent study (ten Cate & Exterkate, 1986), it was reported that during *in vitro* demineralisation studies, more mineral was lost from sections than from bulk enamel. This finding had important implications since this type of specimen was chosen for all studies undertaken in this thesis. It was therefore considered important to repeat the study, comparing the *in vitro* demineralistion of thin enamel sections and bulk enamel.

3.2 Materials and methods

In this study, eight human premolar teeth were used. A three millimeter slab, and one or two sections were cut either from the central aspect of the buccal surface of each tooth or from the mesial or distal ends. The manner in which the specimens were cut is illustrated in Figure 3.1. The sections from each tooth were hand ground to 120 µm. All natural and cut surfaces, on the sections and bulk specimens, were coated with acid-resistant nail varnish, leaving two 300 µm windows on the natural outer enamel. The slab and sections from each tooth were demineralised in a 100 mL aliquot of demineralising buffer containing calcium and phosphate at pH 4.5 as described in Section 2.2.3. At the end of the demineralisation period, the nail varnish was removed from all slabs and sections and

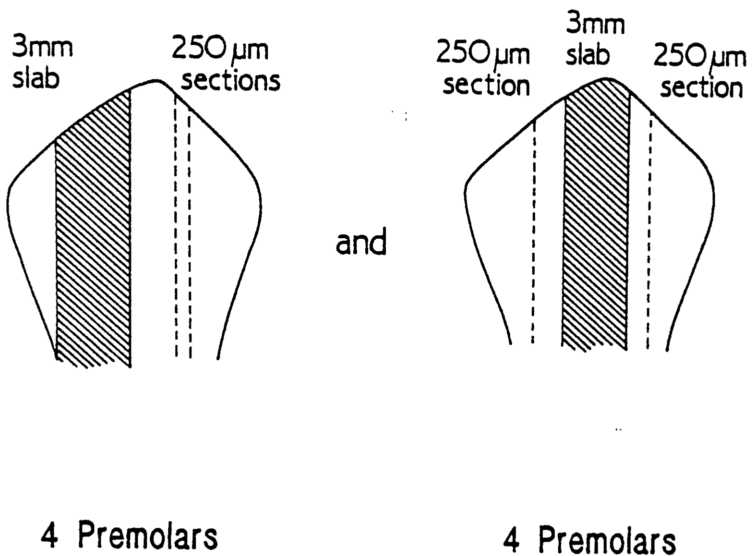


Figure 3.1 **Diagrammatic representation of the buccal surfaces of two premolar teeth showing the segments used for the preparation of section and slab specimens**

radiographs of the sections and of the sectioned slabs were taken. The mineral content of lesions created on both types of enamel specimens were measured by microradiography and microdensitometry and the Δz (total mineral loss) and % volume mineral content of the surface zone (SZ), and lesion body (LB) calculated as described in Section 2.5.4.

3.3 Results

The number of lesions analysed included thirteen from the sections and twelve from bulk enamel. Some lesions could not be assessed accurately because of the curvature of the specimens and were therefore not included in the study. In Table 3.1 the values of the parameters investigated for each lesion are listed. The mean Δz (SE) was 3085 ($\pm$ 293) % vol. min. µm for the sections and 3419 ($\pm$ 330) % vol. min. µm for the slabs. The values for the surface zone were 52.0 ($\pm$ 2.5) and 52.9 ($\pm$ 2.4) % vol. min. for the sections and slabs respectively. The corresponding values for the lesion body were 44.7 ($\pm$ 3.7) and 40.9 ($\pm$ 4.5) % vol. min. No significant differences between sections and slabs were found for any parameter (Student's t-test), the values are illustrated in Figure 3.2. Microdensitometric profiles of lesions created on a section (a), and a slab (b), are shown in Figure 3.3. These profiles show the way in which the % volume mineral content of the lesions vary with depth.

Table 3.1 Δz, surface zone (SZ), lesion body (LB) values after 1 week in vitro demineralisation

	Δz (% vol. min. x µm)	SZ (% vol. min.)	LB (% vol. min.)
Section specimens (n = 13)			
	-2393	60.5	49.8
	-3969	51.9	35.2
	-2631	56.2	54.6
	-2744	49.3	50.2
	-3836	49.1	30.8
	-2249	58.2	60.1
	-2348	51.3	51.2
	-4328	50.1	25.2
	-3602	56.4	41.7
	-2599	52.5	52.1
	-912	68.8	68.6
	-4794	32.3	27.0
	-3709	39.5	35.0
mean	-3085	52.0	44.7
SE	293	2.5	3.7
Slab specimens (n = 12)			
	-2099	62.5	58.7
	-2108	65.1	56.6
	-2710	62.6	48.4
	-3345	47.5	48.5
	-4804	36.8	20.3
	-4299	55.9	26.2
	-2327	56.7	51.6
	-5258	44.2	19.8
	-2828	52.3	53.3
	-4993	45.7	19.9
	-2872	51.2	52.4
	-3383	53.6	34.8
mean	-3419	52.9	40.9
SE	330	2.4	4.5

SE = Standard Error of mean

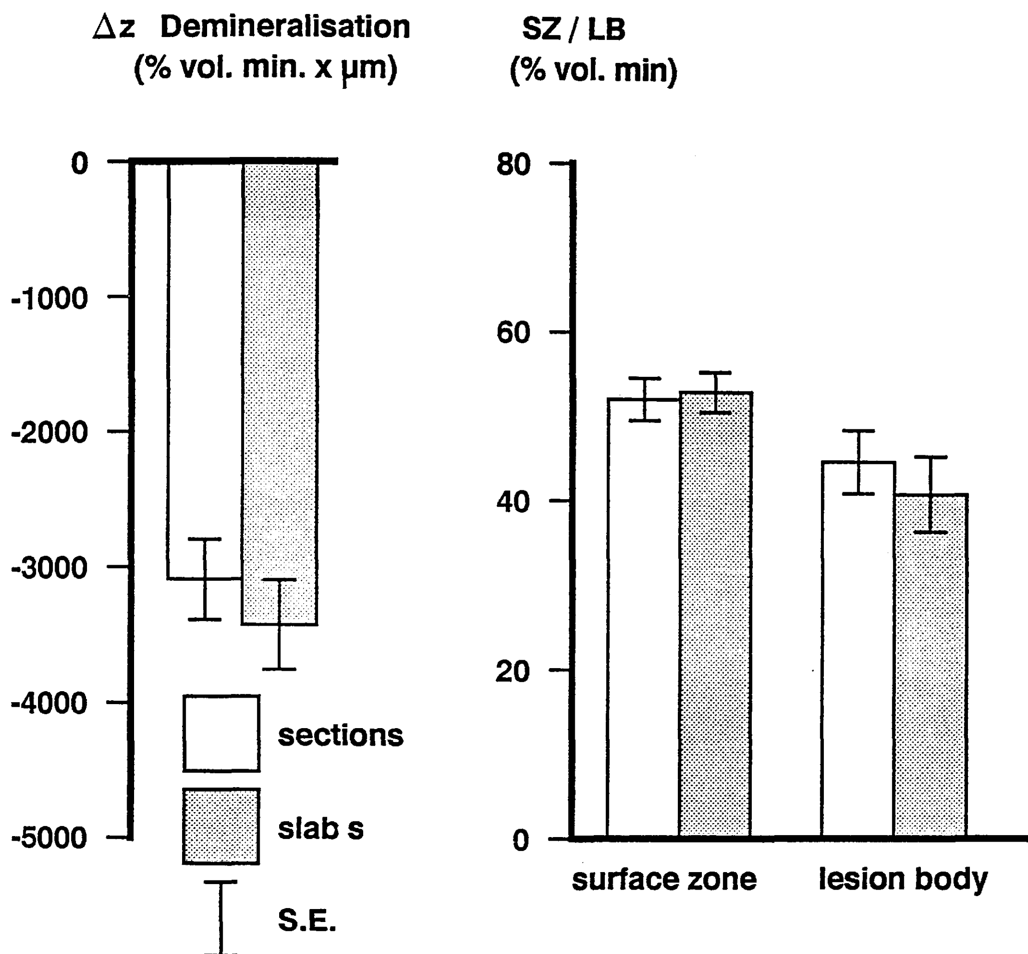
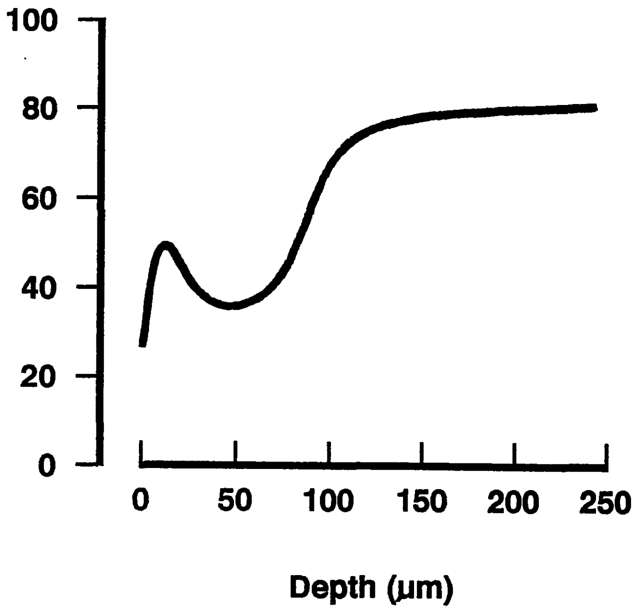


Figure 3.2 Mean Δz, surface zone (SZ) and lesion body (LB) mineral contents for section specimens (n = 13) and slab (bulk) specimens (n = 12)



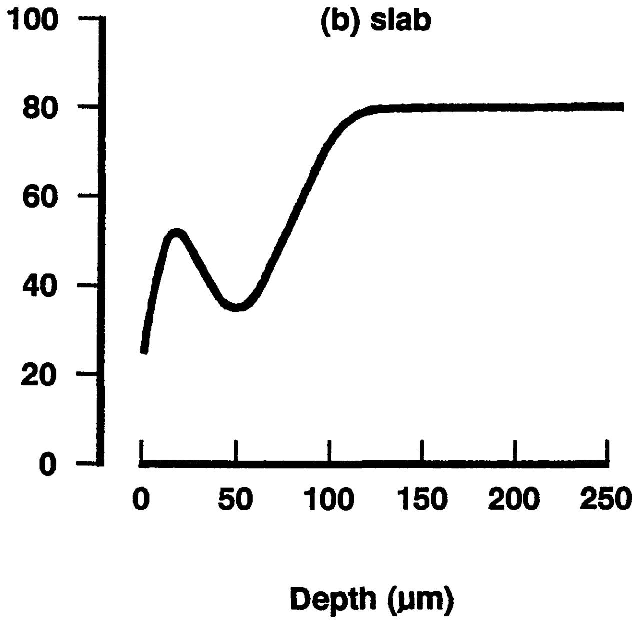


Figure 3.3 Typical microdensitometric profiles of lesions from (a) a section and (b) a slab, specimen

3.4 Discussion

As shown in Figure 3.3 the microdensitometric profiles for the lesions from the section and slab were not identical. Such substantial differences in the shape of lesion profiles are also commonly observed when using lesions derived from section specimens only. These observed differences can be attributed to the natural variation found in human enamel. Nevertheless, averaging over all profiles the mean Δz (% vol. min. x µm), surface zone and lesion body (% vol. min.) parameters were not significantly different.

This study demonstrates that *in vitro* demineralisation of sections and slabs is the same. This result contradicts the findings of other workers (Silverstone *et al.*, 1983., ten Cate & Exterkate, 1986) who suggested that enamel becomes more susceptible to acid attack as a result of vibrations during cutting, with subsequent damaging of enamel prisms. In the present experiment, this problem was avoided by cutting sections at a very slow speed to a thickness of 250 µm and then hand-grinding to the correct final thickness of 120 µm.

Chapter 4

Preliminary studies in the development of a pH cycling model

4.1 Introduction

As described in section 1.6.5, pH cycling models provide useful tools for studying de/- remineralisation processes because they mimic the dynamic *in vivo* situation. The success of a pH cycling model depends on employing optimium conditions in which both demineralisation and remineralisation can take place. If the demineralising cycle is too cariogenic, net demineralisation, or complete dissolution, of the specimens results, giving little information on any subtle changes caused by other factors in the remineralising phase. On the other hand, an inadequate acid challenge results in less fluoride uptake by enamel (Bibby, 1947; Ramsay *et al.*, 1973; Larsen, 1974; ten Cate & Duijsters, 1982). The ideal model is one in which conditions are selected so that both remineralisation and demineralisation of specimens can be measured under experimental conditions.

Ten Cate and Duijsters (1982), ten Cate and Simmons (1986) employed conditions to mimic mild cariogenic conditions. Their model included a three hour demineralisation period using a calcium phosphate buffer at pH 4.7, followed by a 21 hour remineralising phase. Featherstone and

co-workers (1986), used conditions which directly simulated the caries challenge that occurred (during one month) underneath and adjacent to orthodontic brackets. Here specimens were exposed to a calcium phosphate demineralising buffer (pH 4.3) for a period of six hours, after which they were stored in a remineralising solution for the remainder of the day. It was suggested that the latter model represented drastic conditions which would be found only in subjects who snacked frequently.

The Glasgow Caries Research Group attempted to establish an *in vitro* daily acid attack regime, to mimic the *in situ* demineralisation of sections of sound enamel mounted on intraoral appliances (MacDonald *et al.*, 1986). *In vitro* demineralisation was studied by subjecting specimens to a daily routine of eight hours storage in artificial saliva and 16 hours in lactic acid gelatin, using a range of gelatin pH. However, due to problems associated with the accurate measurement of gelatin pH, it was decided to undertake a similar *in vitro* study in which the demineralisation challenge consisted of calcium phosphate solutions of varying hydrogen ion concentrations (Besic, 1953; Coolidge, Besic & Jacobs, 1955; Margolis, Murphy & Moreno, 1985; Theuns *et al.*, 1985; Theuns, Driessens & van Dijk, 1986).

4.2 Materials and methods

Enamel sections were prepared from eighteen human premolar teeth which contained areas of artificial caries and sound enamel. The artificial carious lesions were prepared using the gelatin-system (Section 2.2.3). The ground sections were allocated to different pH cycling regimens as shown in Table 4.1. Each group of sections was exposed to a test solution of different hydrogen ion concentration for either 24 hours or for 16 hours, the latter group being placed in an artificial saliva (Section 2.3) for the remainder of each day. To the demineralising solutions (2mM Ca, 2mM P, 50 mM acetic acid), various amounts of sodium hydroxide were added to provide solutions with a pH range between pH 4 and pH 5. The pH in the resulting solutions was measured to within 0.04 pH units. The calcium and phosphate concentrations were kept fixed, so that the degree of undersaturation with respect to hydroxyapatite was altered by changing the pH of the solution (solutions at pH 4 being very undersaturated and solutions at pH 5 being close to saturation with respect to hydroxyapatite, Theuns et al., 1985). Each section was placed in five mL demineralising solution for the allocated time, solutions being changed daily in order to keep the concentration of ions constant within the limits of experimental error. As discussed in Section 2.3 conditions for the remineralising solution were such that it was stable for at least 24 hours after which fresh remineralising solution was prepared. Weekly, microradiographs were taken of specimens

Table 4.1 pH, hydrogen ion concentration, and daily de- / remineralisation cycling times used in the different experimental groups

pH	$[H^+]$ ($x10^{-5}$)	pH cycling times Demin. (hrs)	Remin. (hrs)
5.2	0.60	16	8
5.0	1.00	24	-
5.0	1.00	16	8
4.8	1.59	16	8
4.6	2.51	24	-
4.6	2.51	16	8
4.4	3.98	16	8
4.2	6.31	16	8
4.0	10.00	24	-
4.0	10.00	16	8

exposed to solutions above pH 4.8, whereas specimens exposed to the pH 4 solutions were microradiographed daily and solutions with a pH between 4.2 and 4.6 were microradiographed every three days.

4.3 Results

Mineral content changes in lesions and adjacent sound enamel were measured and the Δz, surface zone and lesion body de-/ remineralisation rates calculated. Tables 4.2 and 4.3 (summarised in Table 4.6) give the individual and mean ($\pm$ SE) de-/ remineralisation rates for the pH cycled lesions and sound enamel respectively. The corresponding values for the specimens subjected to continuous demineralisation are shown in Tables 4.4 and 4.5 (summarised in Table 4.7).

Cycled groups: The graph in Figure 4.1 shows the change in mean Δz demineralisation rates for the cycled specimens when subjected to the demineralising solutions of varying hydrogen ion concentration. The Δz ($\pm$ SE) values for the lesions ranged from -343 ($\pm$ 305) % vol. min. µm / wk at pH 5 to -4195 ($\pm$ 668) % vol. min. x µm / wk at pH 4 (Table 4.6). The corresponding mean ($\pm$ SE) Δz values for initially sound enamel adjacent to the lesions were -74 ($\pm$ 109) % vol. min. µm / wk for the pH 4.8 demineralising solution and -9343 ($\pm$ 900) % vol. min. µm / wk for the solution at pH 4. The mean ($\pm$ SE) lesion body demineralisation rate value for the pH 5 group was -1.4 ($\pm$ 0.8) % vol. min / wk and for the pH 4 group the value was -38 ($\pm$ 3) % vol. min. / wk. The lesion body

Table 4.2 Individual Δz, surface zone (SZ) & lesion body (LB) de- / remineralisation rates for lesions exposed, daily, for 16 hours to demineralising solutions of different pH levels (pH cycling)

	De- / Remineralisation Rates*					
	Δz (% vol. min. x µm / wk)	SE	SZ (% vol. min. / wk)	SE	LB (% vol. min. / wk)	SE
16 hrs. demin. pH 5.0						
	-950	42	-1.92	0.26	-2.96	0.20
	-77	41	-0.22	0.43	-0.76	0.43
	-2	31	-0.06	0.24	-0.47	0.29
mean	-343	305	-0.73	0.59	-1.40	0.79
16 hrs. demin. pH 4.8						
	-638	84	-1.33	0.48	-3.29	0.28
	-490	105	-1.40	0.49	-2.73	0.35
	-315	112	0.35	0.98	-2.17	0.42
	-273	91	0.14	0.63	-0.49	0.21
	-217	105	0.42	1.05	0.21	0.84
mean	-386	78	-0.36	0.40	-1.69	0.67
16 hrs. demin. pH 4.6						
	-1218	77	0.98	0.35	-4.69	0.42
	-1351	70	1.47	0.70	-6.30	1.40
	-623	168	0.42	0.91	-6.16	1.26
mean	-1064	224	0.95	0.30	-5.72	0.51
16 hrs. demin. pH 4.4						
	-1127	84	-0.49	0.56	-9.24	1.19
	-1120	399	1.12	0.14	-19.2	9.1
	-1414	203	-1.75	0.21	-13.1	3.2
	-1232	392	1.75	0.56	-11.5	5.0
	-1029	504	-0.63	1.82	-9.52	5.7
mean	-1184	66	0.00	0.63	-12.5	1.8
16 hrs. demin. pH 4.2						
	-2597	1239	-37.0	14.0	-39.0	13.0
	-2723	1197	-12.0	8.0	-32.0	9.0
	-3017	527	-17.0	5.0	-33.0	1.0
	-5481	861	-59.0	19.0	-94.0	12.0
mean	-3454	681	-31.0	11.0	-50.0	15.0
16 hrs. demin. pH 4.0						
	-5530	2720	-16.0	13.0	-33.0	18.0
	-3493	791	-14.0	19.0	-44.0	11.0
	-3563	2107	-33.0	17.0	-37.0	15.0
mean	-4195	668	-21.0	16.0	-38.0	3.0

* +ve values = remineralisation
-ve values = demineralisation
SE = Standard Error

Table 4.3 Individual Δz, surface zone (SZ) & lesion body (LB) de- / remineralisation rates for sound enamel exposed, daily, for 16 hours to demineralising solutions of different pH levels (pH cycling)

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x μm / wk)		(% vol. min. / wk)		(% vol. min. / wk)	
16 hrs. demin. pH 4.8						
	-68	19	-0.76	0.03	-0.53	0.04
	-80	14	-0.67	0.20	-0.89	0.15
16 hrs. demin. pH 4.6						
	-910	70	-7.07	1.75	-15.0	13.0
	-364	70	-4.27	1.33	-7.0	1.0
	-427	42	-9.24	2.24	-10.0	2.0
	-945	252	-4.97	0.91	-11.0	3.0
	-147	119	-1.05	0.14	-3.0	1.0
mean	-559	157	-5.32	0.20	-9.0	2.0
16 hrs. demin. pH 4.4						
	-3066	525	-6.65	3.08	-26.0	2.4
	-1512	203	-3.64	0.56	-15.0	1.5
	-4585	637	-17.6	4.62	-51.0	12.4
mean	-3054	887	-9.0	2.00	-32.0	6.0
16 hrs. demin. pH 4.2						
	-3297	924	-73.0	22.4	-78.0	13.0
	-4879	1064	-80.0	36.0	-96.0	34.0
16 hrs. demin. pH 4.0						
	-9016	1750	-110.0	4.0	-119.0	29.0
	-10850	1218	-44.0	20.0	-105.0	14.0
	-6930	119	-78.0	19.0	-106.0	19.0
	-10577	1281	-80.0	62.0	-115.1	49.0
mean	-9343	900	-78.0	50.0	-111.0	30.0

* +ve values = remineralisation
-ve values = demineralisation
SE = Standard Error

Table 4.4 **Individual Δz, surface zone (SZ) & lesion body (LB) de- / remineralisation rates for lesions exposed continuously to demineralising solutions of different pH**

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x µm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
24 hrs. pH 5.0						
	-576	55	-0.01	0.29	-3.04	0.43
	-860	67	-0.52	0.21	-4.03	0.48
	-462	59	-0.39	0.25	-2.01	0.39
	-324	53	-0.01	0.20	-0.95	0.18
mean	-557	113	-0.25	0.12	-2.51	0.66
24 hrs. pH 4.8						
	-700	140	-0.49	0.42	-5.18	0.56
	-686	336	-0.77	2.10	-4.90	1.05
	-21	49	2.73	2.52	1.05	1.47
	-777	308	1.82	2.03	-2.31	0.84
mean	-546	176	0.82	0.86	-2.80	1.45
24 hrs. pH 4.6						
	-910	168	-4.27	0.91	-10.15	1.19
	-763	70	0.70	0.63	-8.05	1.05
	-1043	112	-0.07	0.63	-8.86	0.98
	-924	175	-0.49	0.70	-2.38	0.42
mean	-910	57	-1.19	1.16	-7.32	1.70
24 hrs. pH 4.4						
	-3143	252	-1.82	3.43	-10.36	3.50
	-2128	966	5.04	4.97	-8.19	1.89
	-1687	161	0.98	2.10	-11.20	1.89
	-3528	203	-7.21	5.10	-23.73	2.80
mean	-2621	429	-0.75	2.57	-13.37	3.50
24 hrs. pH 4.2						
	-4725	2366	-33.6	5.0	-53.3	16.7
	-4634	2904	-28.6	15.1	-43.1	22.8
	-7707	1295	-70.1	4.3	-63.2	9.7
	-4382	651	-27.6	6.6	-17.0	5.4
	-2184	1902	-20.1	3.7	-11.8	10.8
mean	-4589	731	-33.5	7.6	-33.0	9.5
24 hrs. pH 4.0						
	-3668	63	4.13	8.4	-11.5	2.8
	-5124	2177	2.03	14.4	-41.2	11.8
	-4501	805	-14.00	9.0	-46.1	10.9
	-7826	3976	-31.08	27.0	-66.5	16.9
	-7546	5040	-52.01	36.7	-69.4	45.9
mean	-5733	831	-18.19	10.6	-46.9	10.4

* +ve values = remineralisation
-ve values = demineralisation
SE = Standard Error

Table 4.5 Individual Δz, surface zone (SZ) & lesion body (LB) de- / remineralisation rates for sound enamel exposed continuously to demineralising solutions of different pH

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x µm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
24 hrs. pH 5.0						
	-520	75	-2.61	0.60	-6.14	0.81
	-234	51	-1.59	0.40	-2.18	0.29
	-262	24	-4.19	0.67	-5.31	0.49
	-227	62	-1.62	1.30	-2.39	1.17
mean	-311	161	-2.50	0.61	-4.05	1.00
24 hrs. pH 4.8						
	-966	126	-9.24	1.82	-16.0	0.63
	-1057	119	-5.25	3.08	-15.5	1.82
	-791	210	-3.57	3.22	-12.2	1.26
	-1113	147	-4.55	2.17	-15.1	2.03
	-1330	28	-14.8	5.95	-20.6	6.86
	-903	231	-11.6	4.83	-11.4	6.23
mean	-1027	200	-8.18	1.83	-15.1	1.33
24 hrs. pH 4.6						
	-1099	105	-2.80	0.49	-9.1	0.49
	-2387	77	-3.08	1.75	-16.4	3.43
	-1113	371	-9.10	1.82	-13.2	2.17
	-1687	350	-11.3	2.38	-13.9	2.13
mean	-1568	78	-6.58	2.14	-13.2	1.50
24 hrs. pH 4.4						
	-3192	343	-5.18	2.31	-34.8	3.6
	-3479	749	-6.16	5.25	-35.3	4.4
	-2114	322	-9.52	3.78	-35.3	8.2
	-3801	1015	-10.5	17.7	-42.4	7.6
mean	-3146	168	-7.84	1.28	-36.9	1.8
24 hrs. pH 4.2						
	-9387	3458	-110.6	5.6	-175.0	17.2
	-7063	1771	-31.8	2.5	-65.3	9.6
	-3563	235	-101.1	41.6	-99.6	43.5
	-3283	4263	-61.4	17.2	-76.7	33.0
	-6825	2352	-68.0	12.0	-71.4	14.1
	-7427	1708	-85.9	10.9	-77.1	10.3
mean	-6258	411	-76.5	11.8	-94.2	17.0
24 hrs. pH 4.0						
	-6650	1358	-65.6	23.0	-100.0	33.7
	-10136	413	-35.0	32.6	-110.0	27.9
	-7742	2072	-62.2	15.8	-126.0	5.5
	-10388	1967	-88.8	13.0	-147.0	19.3
mean	-8729	380	-62.9	11.0	-121.0	10.0

* +ve values = remineralisation, -ve values = demineralisation
SE = Standard Error

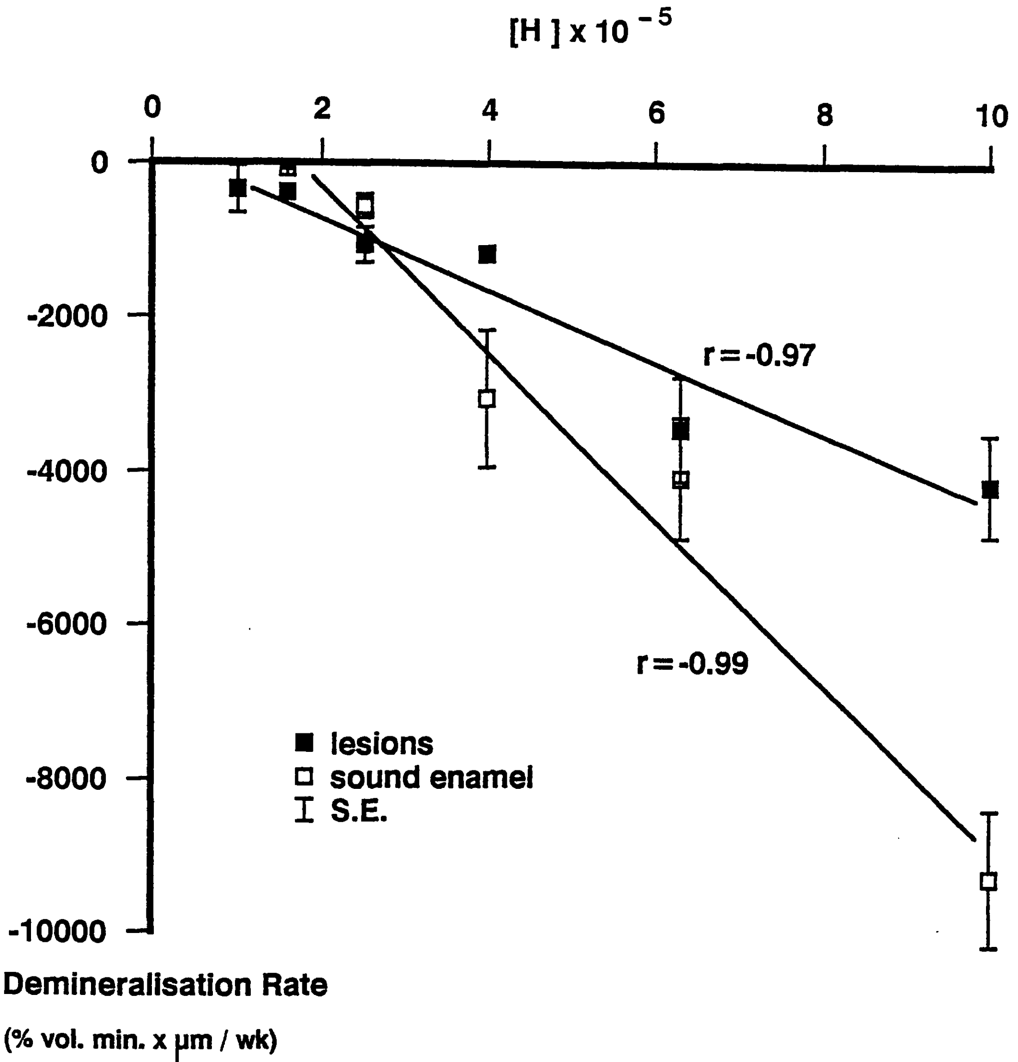


Figure 4.1 Variation in Δz demineralisation rates with hydrogen ion concentration for specimens subjected to 16 hours demineralisation and eight hours remineralisation

Table 4.6 Mean (SE) Δz, surface zone (SZ) & lesion body (LB) de- / remineralisation rates for (a) lesions, and (b) initially sound enamel, exposed, daily, for 16 hours to demineralising solutions of different pH levels (pH cycling) Summary of Table 4.2 and 4.3

pH	De- / Remineralisation Rates*		
	Δz (% vol. min. x μm / wk)	SZ (% vol. min. / wk)	LB (% vol. min. / wk)
(a) Lesions (Table 4.2)			
5.0	-343 (305)	-0.73 (0.59)	-1.40 (0.79)
4.8	-386 (78)	-0.36 (0.40)	-1.69 (0.67)
4.6	-1064 (224)	0.95 (0.30)	-5.72 (0.50)
4.4	-1184 (66)	0.00 (0.63)	-12.5 (1.8)
4.2	-3454 (681)	-31.00 (11.0)	-50.0 (15.0)
4.0	-4195 (668)	-21.00 (16.0)	-38.0 (3.0)
(b) Initially sound enamel (Table 4.3)			
4.8	-74 (10)	-0.77 (0.15)	0.70 (0.10)
4.6	-559 (157)	-5.32 (0.20)	-9.00 (2.0)
4.4	-3054 (887)	-9.00 (2.0)	-32.0 (6.0)
4.2	-4088 (791)	-76.00 (25.0)	-87.0 (20.0)
4.0	-9343 (900)	-78.00 (50.0)	-111.0 (30.0)

* +ve values = remineralisation
-ve values = demineralisation
SE = Standard Error

demineralisation rate value of the adjacent enamel was -0.7 (± 0.1) % vol. min. / wk for the pH 4.8 group and -111 (± 30)% vol. min. / wk for the pH 4 group. The mean (± SE) surface zone mineralisation rates of the pre-formed lesions ranged from -0.73 (± 0.59) % vol. min. / wk at pH 4 to -21 (± 16) % vol. min / wk at pH 5 whereas the corresponding values for the initially sound enamel varied from -0.77 (± 0.15) to -78 (± 50) % vol. min. / wk.

Continuously demineralised groups: Figure 4.2 shows how the mean Δz demineralisation rates for the continuously demineralised groups was affected by changes in the hydrogen ion concentration of the demineralising solution. As expected, the values were higher than the corresponding values obtained for the pH cycled groups. Lesions showed a mean Δz (± SE) demineralisation rate of -557 (± 113) % vol. min. µm / wk at pH 5, increasing to -5733 (± 831) % vol. min. µm / wk at pH 4. As shown in Table 4.7, the initially sound enamel again demineralised faster than the carious enamel giving a mean Δz (± SE) rate of -311 (± 161) % vol. min. µm / wk at pH 5 and increasing rapidly to -8729 (± 380) % vol. min. µm / wk at pH 4. The corresponding mean lesion body values for the pre-formed lesions were -2.51 (± 0.66) % vol. min. / wk at pH 5 and -46.9 (± 10.4) % vol min. / wk at pH 4. For the adjacent (initially sound) enamel the values were -4 (± 1) % vol. min. / wk at pH 5 and -121 (± 10) % vol. min. / wk at pH 4. The surface zone values for the pre-formed lesions ranged from -0.25 (± 0.12) % vol. min / wk at pH 5

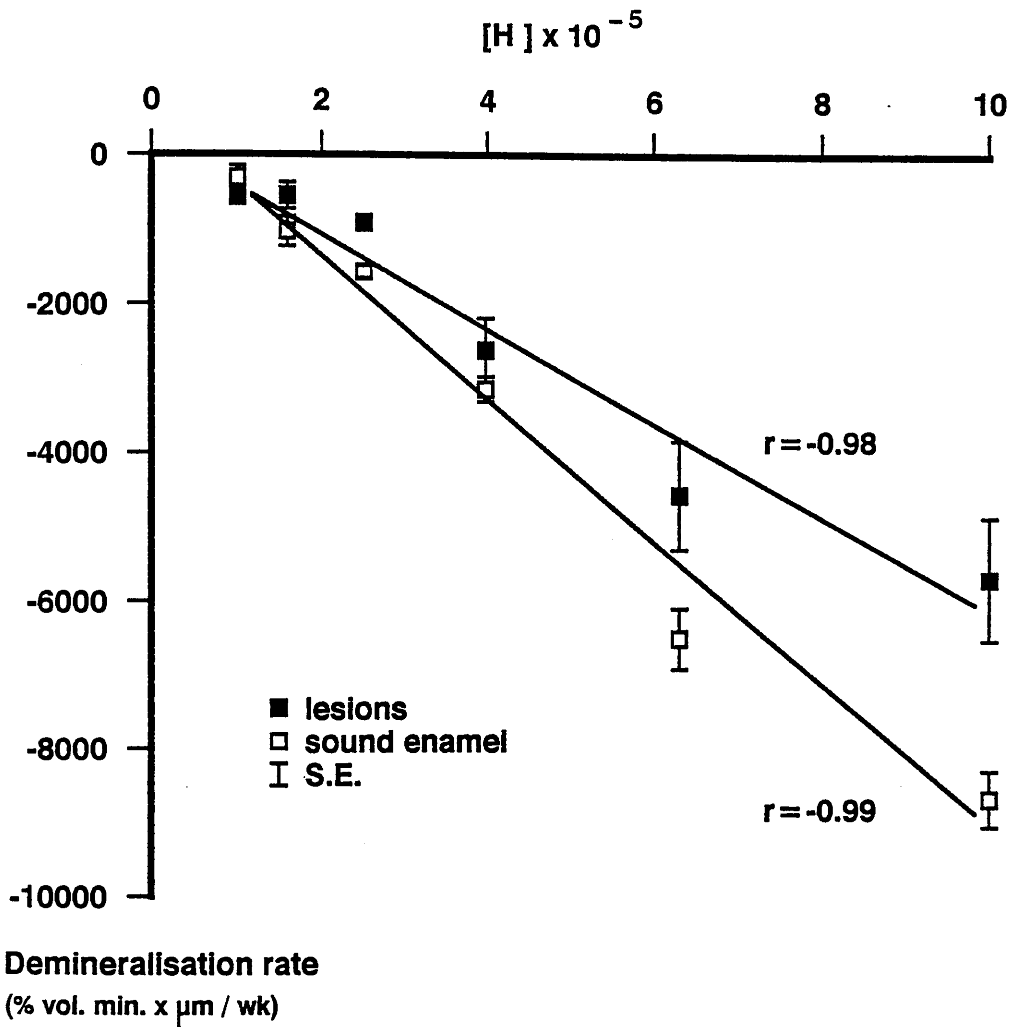


Figure 4.2 Variation in Δz demineralisation rates with hydrogen ion concentration for specimens subjected to continuous demineralisation

Table 4.7 Mean (SE) Δz, surface zone (SZ) & lesion body (LB) de- / remineralisation rates for (a) lesions, and (b) initially sound enamel exposed continuously to demineralising solutions of different pH
Summary of Table 4.4 and 4.5

pH	De- / Remineralisation Rates* Δz (% vol. min. x µm / wk)	SZ (% vol. min. / wk)	LB (% vol. min. / wk)
(a) Lesions (Table 4.4)			
5.0	-557 (113)	-0.25 (0.12)	-2.51 (0.66)
4.8	-546 (176)	0.82 (0.86)	-2.80 (1.45)
4.6	-910 (57)	-1.19 (1.16)	-7.32 (1.70)
4.4	-2621 (429)	-0.75 (2.57)	-13.4 (3.5)
4.2	-4589 (731)	-33.5 (7.6)	-33.0 (9.5)
4.0	-5733 (831)	-18.2 (10.6)	-46.9 (10.4)
(b) Initially sound enamel (Table 4.5)			
5.0	-311 (161)	-2.50 (0.61)	-4.05 (1.00)
4.8	-1027 (200)	-8.18 (1.83)	-15.1 (1.3)
4.6	-1568 (78)	-6.58 (2.14)	-13.2 (1.5)
4.4	-3146 (168)	-7.84 (1.28)	-36.9 (1.8)
4.2	-6258 (411)	-76.5 (11.8)	-94.2 (17.0)
4.0	-8729 (300)	-62.9 (11.0)	-121.0 (10.0)

* +ve values = remineralisation
-ve values = demineralisation
SE = Standard Error of mean

to -18.2 (± 10.6) % vol. min. / wk at pH 4 whereas the initially sound enamel gave a value of -2.5 (± 0.61) % vol. min. / wk at pH 5 and a value of -62.9 (11) % vol. min. / wk at pH 4.

4.4 Discussion

The driving force for enamel dissolution is dependant on all ions present in a demineralising medium (Margolis, Murphy & Moreno, 1985). In this study, however, the demineralising solutions all contained the same amount of calcium and phosphate, irrespective of the hydrogen ion concentration. Hence the degree of saturation of the solution with respect to the enamel mineral, and therefore the driving force for enamel dissolution, depended on the concentration of hydrogen ions.

The lower rate of demineralisation of the pre-formed lesions compared to the adjacent, initially sound enamel is explained by the presence of high fluoride levels which are present in carious enamel (Dowse & Jenkins, 1957; Little, Posen & Singer, 1962; Koulourides & Cameron, 1980). Under the conditions of the experiment enamel dissolution increased dramatically with decrease in pH and duration of the acid challenge.

This study showed that it is possible to achieve variable rates of enamel demineralisation by using well-defined calcium phosphate solutions. Solutions with the same calcium and phosphate concentration but with a pH of less

than 4.8 were excessively cariogenic since dissolution of the specimens occurred too rapidly. The demineralisation rates obtained with solutions of pH 4.8 and pH 5 respectively compared well with those reported in an investigation on *in vitro* and *in situ* demineralisation (MacDonald *et al.,* 1986). These solutions should therefore be suitable for *in vitro* studies requiring an acid challenge similar to that which occurs *in situ.*

Chapter 5

Comparison of solution- and gelatin-prepared lesions - A pH-cycling experiment

5.1 Introduction

In vitro techniques for the production of artificial carious lesions have been used since 1867 when Magitot demonstrated that fermentation of sugars caused dissolution of teeth. Since then numerous artificial lesion producing systems (Section 1.6.4) have been developed and used to obtain information on de-/remineralisation processes. It has been suggested (Mellberg & Chomicki, 1983), that artificial lesions prepared by different systems, may respond differently when subjected to a series of experimental protocols. For instance, lesions prepared rapidly are advantageous when testing anticaries agents *in vitro* (Margolis, Murphy & Moreno, 1985), whereas the use of a system employing a mild cariogenic challenge seems preferable in some experimental designs, e.g. in searching for differences in enamel susceptibility of various groups of teeth (Kotsanos *et al.*, 1989). In developing a pH model, there was clearly a need to select artificial lesions that would respond efficiently to different experimental protocols.

A major problem with the acidified gel system is the difficultly in obtaining standardised lesions. This is due

to variations in gel batch composition and also to the presence of impurities. Some of the innate impurities include calcium, phosphate and fluoride ions and the variation in concentration of these ions in different gel batches has been found to be significant (Kotsanos *et al.*, 1989). Many studies measure fluoride uptake and therefore its presence is not desirable because pre-adsorbed fluoride during lesion formation changes the affinity for precipitation (ten Cate & Rempt, 1986). Feagin, Clarkston & Wefel (1985) dialysed the gelatin in order to remove impurities, however, this resulted in decalcification of the surface layer.

Calcium phosphate buffered solutions provide well-defined chemical systems for the production of standardized caries-like lesions. The rate of demineralisation can be easily controlled by altering the degree of saturation of the solution with respect to hydroxyapatite. In addition, this method of artificial lesion preparation is simple and quick and produces lesions with good intact surfaces.

5.2 Aims

The aim of this study was to determine a lesion producing system that would create responsive lesions for *in vitro* and *in situ* studies. This was done by comparing the behaviour of lesions prepared by two methods when subjected to *in vitro* de-/ remineralisation.

In addition, the cariostatic effect of fluoride on these

lesions was also studied by adding 2 ppm fluoride as sodium fluoride to the remineralising solution.

5.3 Materials and methods

Sixteen human premolar teeth were obtained from a region with a low (<0.02 ppm) concentration of fluoride in the drinking water. After cleaning, artificial carious lesions were prepared by two methods. Eight teeth had artificial lesions prepared by the gelatin method (Section 2.2.3) and artificial lesions, on the other eight teeth, were created by immersing the teeth in a buffered solution for five days. In this case the buffer system contained 8.1 mM calcium chloride, 8.1 mM sodium dihydrogen orthophosphate, 50 mM glacial acetic acid, at pH 4.0, pI = 126 (Theuns *et al.*, 1985). These concentrations give the same ion activity product as the demineralising solution described in Section 2.2.3. Thereafter, the teeth were sectioned and three sections (12 lesions) allocated to each of four groups (Table 5.1). Groups A and B contained lactate/gelatin-prepared lesions (Section 2.2.3 (1)) whereas Groups C and D contained buffered acetate/solution-prepared lesions (Section 2.2.3 (2)). All sections were radiographed and later varnished, keeping exposed the normal outer enamel. Each section was then placed in 5 mL of the demineralising solution for 16 hours per day. After rinsing with double-distilled de-ionised water, the sections were placed in the corresponding remineralising solution for the remainder of the day. In Groups B and D, 2 ppm fluoride were added

Table 5.1 Section allocation and pH cycling regimes

Group	Lesion Preparation	Daily Regime
A	gelatin/Lactate	16 hr Demin + 8 hr Remin I
B	gelatin	16 hr Demin + 8 hr Remin II
C	solution/Acetate	16 hr Demin + 8 hr Remin I
D	solution	16 hr Demin + 8 hr Remin II

Demin : 2 mM Ca, 2 mM P, 50 mM acetic acid, pH 4.8

Remin I : 2 mM Ca, 2 mM P, 0.03 ppm F, pH 6.85

Remin II : 2 mM Ca, 2 mM P, 2 ppm F, pH 6.85

to the remineralising solution. Fresh solutions were used daily, the remineralising solution being prepared as discussed in Section 2.3. The enamel sections were cycled in this manner for a period of six weeks, during which radiographs of the unvarnished sections were taken at two day intervals for the first week, and thereafter, once per week.

Measurements of Δz (total mineral loss), surface zone (SZ), and lesion body (LB) mineral contents were obtained for each lesion, and the de- /remineralisation rates calculated as shown in Section 2.5.4.

5.4 Results

The Δz, surface zone and lesion body de-/ remineralisation rates for each lesion in all groups are listed in Table 5.2 and the mean ($\pm$ SE) mineralisation rates for the Δz parameter for the cycled groups are illustrated in Figure 5.1. The demineralisation rates of the solution-prepared lesions (Group C & D) were significantly greater than those of the corresponding gelatin-prepared lesions (Group A & B) viz: (A vs C:- $0.01<p<0.02$; B vs D:- $0.02<p<0.05$, by 2-tailed 't'tests). For both types of lesions, the mean demineralisation rates for the 2 ppm fluoride groups (B & D), were lower than the corresponding values for the non-F groups, although this was not statistically significant.

The mean surface zone and lesion body mineralisation rates for Group A - Group D are illustrated in Figures 5.2 and

Table 5.2 Effect of fluoride concentration and lesion preparation method on Δz, surface zone (SZ), lesion body (LB) de- / remineralisation rates (pH cycling: 8 hrs demin., 16 hrs remin. + F)

Group A (gelatin-prepared lesions: 0 ppm F) n = 11

	De- / Remineralisation Rates*					
	Δz (% vol. min. x µm / wk)	SE	SZ (% vol. min. / wk)	SE	LB (% vol. min. / wk)	SE
	-445	62	-0.92	0.37	-1.25	0.27
	-359	86	-0.45	0.49	-1.04	0.58
	-509	69	-0.08	0.47	-1.10	0.37
	-568	30	0.03	0.26	-2.66	0.63
	-476	64	0.10	0.24	-1.21	0.76
	-232	41	-0.12	0.18	-0.83	0.25
	-117	33	0.28	0.31	-0.57	0.25
	-221	40	-0.10	0.11	-0.98	0.24
	-163	134	0.55	0.61	-1.35	0.81
	-232	68	0.34	0.39	-0.23	0.43
	-1050	58	-0.39	0.26	-2.94	0.62
mean	-398	80	-0.07	0.12	-1.29	0.25

Group B (gelatin-prepared lesions: 2 ppm F) n = 8

	De- / Remineralisation Rates*					
	Δz (% vol. min. x µm / wk)	SE	SZ (% vol. min. / wk)	SE	LB (% vol. min. / wk)	SE
	-256	76	-0.46	0.45	-0.66	0.44
	-143	50	0.71	0.43	-0.14	0.33
	-135	79	-0.27	0.49	-0.60	0.38
	-114	48	-0.35	0.44	-0.36	0.28
	-268	16	-0.35	0.36	-0.44	0.20
	-407	28	-0.69	0.32	-0.95	0.20
	-436	32	0.07	0.30	-0.36	0.38
	-266	50	-0.26	0.33	-1.19	0.29
mean	-253	43	-0.20	0.15	-0.58	0.12

* +ve values = remineralisation
-ve values = demineralisation
SE = Standard Error

Table 5.2 (continued)

Group C (solution-prepared lesions: 0 ppm F) n = 6

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x μm / wk)		(% vol. min. / wk)		(% vol. min. / wk)	
	-526	67	-0.98	0.38	-1.75	0.45
	-188	31	-0.09	0.20	-0.96	0.23
	-109	57	0.06	0.17	-0.41	0.27
	-1391	72	-0.11	0.48	-3.93	0.36
	-1594	104	0.33	0.19	-2.83	0.25
	-1346	110	0.63	0.48	-1.93	0.20
mean	-859	270	0.03	0.22	-1.97	0.52

Group D (solution-prepared lesions: 2 ppm F) n = 7

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x μm / wk)		(% vol. min. / wk)		(% vol. min. / wk)	
	-246	64	0.58	0.54	-0.22	0.36
	-314	65	0.14	0.40	-1.41	0.41
	-394	79	0.75	0.33	-2.05	0.58
	-421	58	1.24	0.27	-1.68	0.40
	-877	86	-0.95	0.45	-3.66	0.97
	-823	61	2.27	0.36	-3.75	0.83
	-1259	103	-0.29	0.52	-1.10	0.37
mean	-619	141	0.53	0.40	-1.98	0.49

* +ve values = remineralisation
-ve values = demineralisation
SE = Standard Error

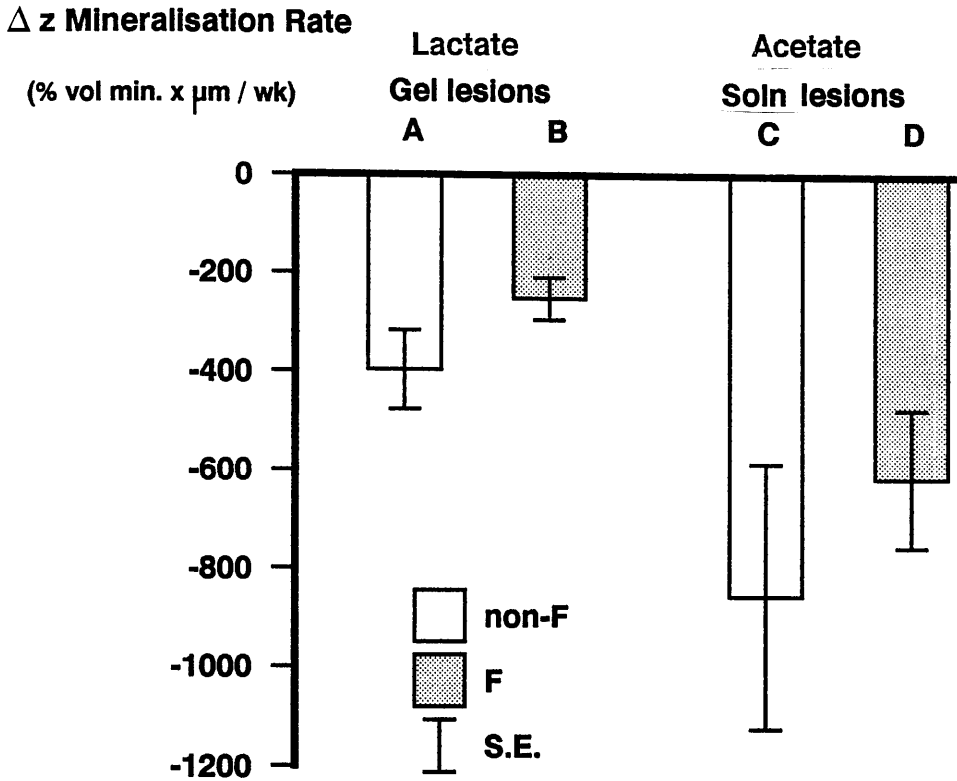


Figure 5.1 Mean Δz demineralisation rates for the four Groups A, B, C and D
"Gel" - gelatin-prepared lesions (A & B)
"Soln" - solution-prepared lesions (C & D)

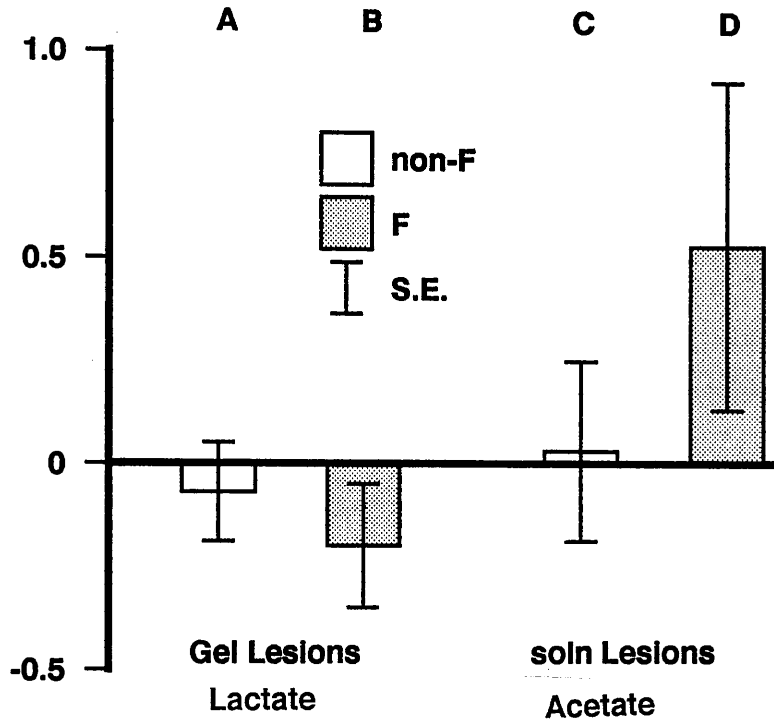


Figure 5.2 Mean surface zone (SZ) de-/remineralisation rates for the four Groups A, B, C and D
"Gel" - gelatin-prepared lesions (A & B)
Soln." - solution-prepared lesions (C & D)

5.3 respectively. Only the solution-prepared lesions of the sections exposed to fluoride (Group D) showed an increase in mineral content of the surface zone. This value was significantly different ($p = 0.05$) from the corresponding value for the gelatin-prepared lesions (Group B). However, no statistically significant differences were noted between Groups A & B (gelatin lesions: non-F vs F), Groups A & C (non-F: gelatin vs solution) and Groups C & D (solution lesions: non-F vs F). On the other hand, for the lesion body parameter, addition of fluoride to the remineralising solution, significantly decreased the demineralisation rate for the gelatin-prepared lesions. (A vs B: $0.02<p<0.05$), but had no effect for solution-prepared lesions. For the non-F groups, Group C was not significiantly different from Group A, whereas for the fluoride groups, the solution-prepared lesions (Group D) demineralised significantly more than did the gelatin-prepared Group B lesions, ($0.01<p<0.02$).

All the sections not exposed to fluoride in the remineralising solution showed subsurface demineralisation in initially sound enamel adjacent to the lesions. In contrast, only one area of sound enamel on one of the sections exposed to the remineralising solution which contained fluoride showed signs of mineral loss.

A feature of this study was the appearance of laminations. These were apparent in the mineral content

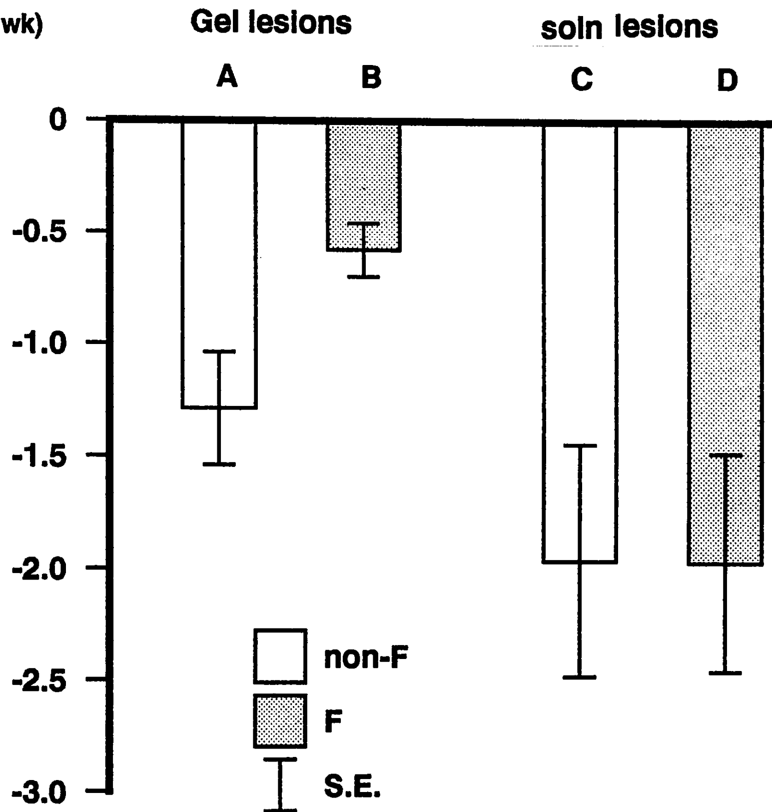


Figure 5.3 Mean lesion body (LB) demineralisation rates for the four Groups A, B, C, D
"Gel" - gelatin-prepared lesions (A & B)
"Soln" - solution-prepared lesions (C & D)

profiles (Fig. 5.4) of lesions belonging to Group D (solution-prepared lesions with fluoride in the remineralising solution) after two days of cycling. As stated above, only lesions in this group showed a net increase in the % volume mineral content of the surface zone. As can be seen from Figure 5.4, this was accompanied by an increase in lesion depth. No laminations were apparent in the lesions of any other groups.

5.5 Discussion

The results of this study suggest that the method of artificial carious lesion preparation affects the behaviour of such lesions when they are exposed to a demineralising and remineralising protocol. Lesions prepared by an acid buffered solution demineralised to a greater extent than did crude gelatin-prepared lesions. This finding suggests that the high mineral ion impurity of crude gelatin might interfere with the processes taking place. In addition, undialysed gelatin contained levels of fluoride in the region of 0.15 ppm. Caries-like lesions prepared in such gels have very high fluoride levels (Clarkston, Wefel & Feagin, 1986). These lesions, whilst still in the gelatin medium, could therefore have reached a stage of "arrestment" as a result of preferential surface layer deposition. Indeed scanning electron microscopy has shown that there is a large difference in surface porosity between active and arrested lesions (Thylstrup & Fredebo, 1982). This

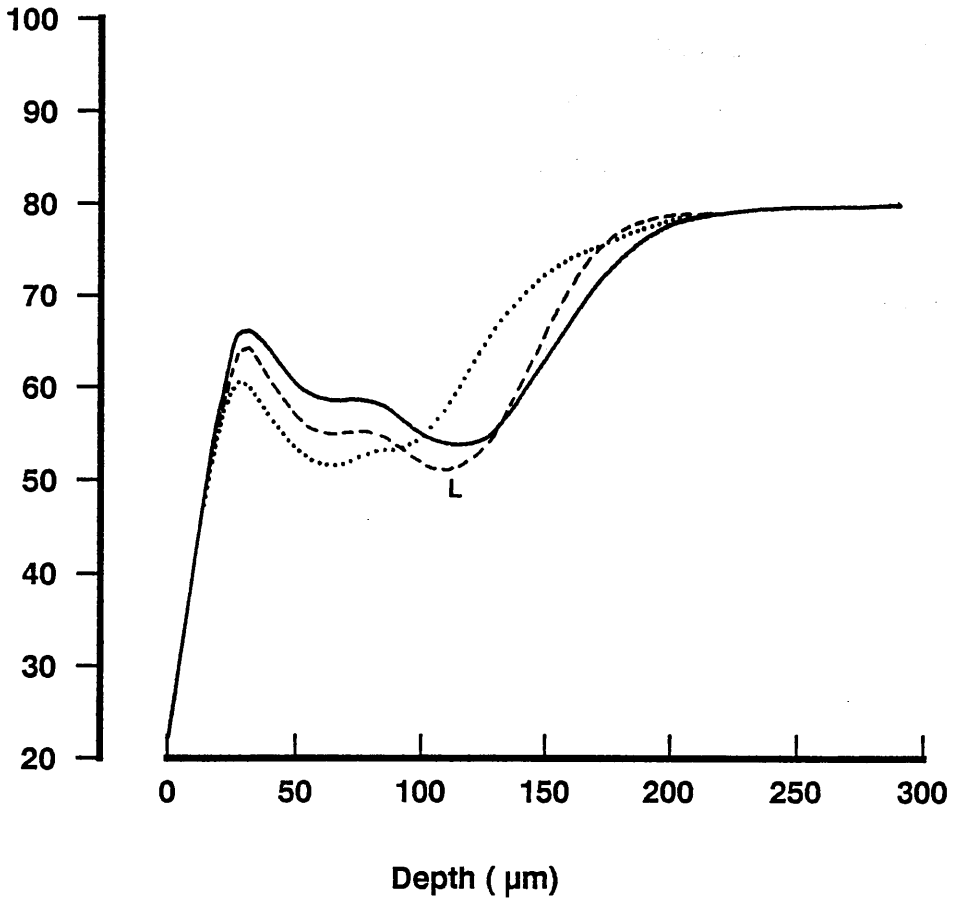


Figure 5.4 Mineral content profiles of a lesion from Group D (solution-prepared lesion with F) after four weeks (....) line, five weeks (----) and six weeks (____). An area of lamination is indicated (L).

could account for the greater resistance of the gelatin-prepared lesions to acid attack, when compared with the solution-prepared lesions which were formed in a solution containing less than 0.03 ppm fluoride.

The results of this study clearly showed that lesions prepared by a well-defined buffered system were more responsive towards de-/ remineralisation and therefore better for *in vitro* and *in situ* studies.

A number of observations in the work described in this chapter clearly showed that fluoride was exhibiting its cariostatic effects. The sound enamel of the fluoride-treated groups remained resistant to the acid challenge throughout the experiment. Only the lesions in Group D showed laminations. Thus, in view of the fact that the baseline microradiographic parameters of this group did not differ significantly from those of the other groups, it would appear that fluoride was reaching the lesion body, despite the fact that mineral was still being lost from the deeper aspects of the lesion. In this case it is reasonable to suppose that laminations were formed as a result of lesions being treated with fluoride and then being re-exposed to the artificial caries system (Koulourides, 1981; Sato & Yamamoto, 1986).

Chapter 6

Effect of continuous low levels of fluoride - A pH-cycling experiment

6.1 Introduction

Several studies (Koulourides, Cueto & Pigman, 1961; Feagin *et al.*, 1971; Moreno & Zahradnik, 1979; Joyston-Bechal & Kidd, 1982; ten Cate & Duijsters, 1983a, Featherstone *et al .*, 1986) support the hypothesis that daily use of low fluoride levels is required to elevate salivary and plaque fluoride concentrations and to provide fluoride at the time of acid challenge and subsequent remineralisation.

The aim of this study was to demonstrate enhanced remineralisation of artificial lesions when low fluoride concentrations were added to the remineralising phase of a pH-cycling experiment. The fluoride content of the solutions ranged from < 0.03 to 0.5 ppm. Such concentrations were selected because studies have indicated that fluoride levels as low as 0.1 ppm may be sufficient to enhance apatite crystal growth (Amjad & Nancollas, 1979). In addition, although normal levels of salivary fluoride are in the order of 0.01 to 0.03 ppm, these levels are much higher after a fluoride treatment and remain elevated for several hours (Aasenden, Brudevold & Richardson, 1968; Bruun & Givskov, 1979; Brunn *et al.*, 1982; Duckworth, Morgan & Murray, 1987). The pH

cycling protocol chosen pertained to a situation similar to drinking fluoridated water, or to periods in between toothbrushing, or fluoride mouthrinsing.

6.2 Experimental situation

In the pH cycling experiment described in Chapter 5, a daily acid challenge of 16 hours (pH 4.8) followed by a remineralising phase of eight hours, resulted in net demineralisation of all specimens. In order to demonstrate inhibition of demineralisation as well as enhanced remineralisation, it was necessary to limit the demineralising cycle to a shorter duration. Ten Cate and Duijsters (1983a) based the timing of the acid challenge in their pH cycling model on intraoral pH determinations which showed that subcritical pH values of 4.7 - 5.3 occur for two to seven hours per day (Jenkins, 1978). It was therefore decided to limit the daily acid challenge to three hours so that lesion remineralisation and its magnitude with fluoride concentration could be investigated.

This study also utilized solution- and gelatin-prepared artificial lesions, in order that their response under low fluoride conditions could be investigated.

6.3 Materials and methods

Twenty four human premolar teeth extracted for orthodontic reasons were used. The teeth were cleaned and artificial carious lesions again prepared as detailed in Section

2.2.3. Half the teeth had artificial carious lesions prepared by the gelatin method and the other 12 teeth had lesions prepared by the acid buffered system. Longitudinal sections were cut, ground, radiographed and varnished. Sections were then allocated to six groups, each group containing a maximum of 12 gelatin-prepared lesions and 16 solution-prepared lesions at the start of the experiment (Table 6.1). Group A lesions were demineralised continuously and acted as a negative control. Lesions on sections in groups B, C, D, E and F, were demineralised for three hours and remineralised for 21 hours per day. Between each cycle the sections were rinsed in double-distilled de-ionised water and dabbed dry to avoid carry-over of any liquid. The remineralising solution (2 mM $CaCl_2$, 2 mM NaH_2PO_4, pH 6.85) was prepared daily from stock solutions (Section 2.3) and contained fluoride (as sodium fluoride) in concentrations ranging from 0.03 - 0.5 ppm. Cycling of the sections was carried out continuously for five weeks, except for one day per week when the sections were radiographed as described in Section 2.5.2. The de-/ remineralisation rates of lesions was assessed using microdensitometry (Section 2.5.4).

6.4 Results

The results of Δz, surface zone (SZ) and lesion body (LB) de- / remineralisation rates for the solution-prepared lesions and the gelatin-prepared lesions are tabulated in Table 6.2 and Table 6.3 respectively.

Table 6.1 Group allocation for pH cycling study

Group	No. of lesions* "gel"	No. of lesions* "soln"	Daily pH cycle demin. (hrs)	Daily pH cycle remin. (hrs)	remin. ppm F
A	5	7	24	-	<0.03
B	9	10	3	21	<0.03
C	10	12	3	21	0.06
D	9	11	3	21	0.09
E	11	16	3	21	0.12
F	6	12	3	21	0.50

Demineralisation solution:
2 mM Ca, 2 mM P,
50 mM acetic acid,
NaOH to pH 4.8

Remineralisation solution:
2 mM Ca, 2mM P,
F - as above,
NaOH to pH 6.85

* Number of lesions available for analysis at the end of the experiment.

"gel" = gelatin-prepared lesions
"soln" = solution-prepared lesions

Table 6.2 Effect of low fluoride levels on Δz, surface zone (SZ), lesion body (LB) de- / remineralisation rates in a pH cycling study (3 hrs demin., 21 hrs remin. + F). Solution-prepared lesions

Group A (demineralisation only, n = 7)

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x µm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
	-2325	279	-0.78	1.23	-6.04	0.94
	-3059	169	0.01	0.74	-7.23	0.94
	-3369	128	-1.59	1.26	-6.02	0.93
	-1109	115	0.12	0.63	-5.89	1.06
	-2088	633	-2.94	3.0	-3.42	0.04
	-3367	146	-2.68	1.15	-7.21	0.77
	-1715	96	-1.21	0.85	-5.96	0.81
mean	-2433	329	-1.30	0.45	-5.97	0.48

Group B (F $\leqslant$ 0.03 ppm, n =10)

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x µm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
	-15	29	0.01	0.52	0.31	0.22
	-2	24	0.50	0.41	-0.40	0.30
	-123	75	0.63	1.20	-0.88	0.44
	31	36	0.37	0.27	0.30	0.16
	-84	36	-0.78	0.36	-1.14	0.28
	-22	17	0.13	0.26	-0.41	0.37
	-63	58	-0.85	0.62	-0.66	1.00
	49	30	0.81	0.27	-0.21	0.11
	26	39	0.10	0.73	-0.13	0.44
	-22	35	0.12	0.39	-0.03	0.11
mean	-23	17	0.10	0.17	-0.32	0.15

* +ve values = remineralisation
-ve values = demineralisation
SE = Standard Error

Table 6.2 (continued)

Group C (F = 0.06 ppm, n = 12)

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x µm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
	4	88	-0.24	1.04	0.21	0.60
	23	29	-0.95	0.67	0.33	0.13
	-71	43	0.47	0.50	-0.66	0.39
	22	33	1.17	0.58	0.07	0.46
	5	64	1.04	0.56	-0.09	0.84
	-140	111	-0.93	0.62	-0.32	0.92
	-1	27	0.61	0.12	-0.33	0.37
	-41	55	-0.45	0.88	-0.40	0.59
	26	30	0.84	0.28	0.11	0.19
	21	46	0.76	0.31	0.00	0.53
	-1	24	-0.30	0.27	-0.14	0.33
	-6	38	-0.06	0.61	0.32	0.43
mean	14	14	-0.16	0.54	-0.08	0.09

Group D (F = 0.09 ppm, n = 11)

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x µm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
	18	34	0.55	0.44	0.02	0.30
	103	14	0.29	0.61	-0.35	0.30
	20	39	0.12	0.27	0.39	0.26
	-27	36	0.25	0.59	-0.27	0.14
	-26	40	-0.23	0.60	-0.18	0.33
	-6	23	0.52	0.59	-0.02	0.44
	-39	39	-0.54	0.40	-0.46	0.34
	2	30	0.39	0.62	0.08	0.32
	1	36	0.57	0.42	0.12	0.37
	-36	34	-0.07	0.41	0.19	0.29
	-49	55	-0.75	1.17	0.02	0.35
mean	-4	13	0.10	0.14	-0.04	0.08

* +ve values = remineralisation
-ve values = demineralisation
SE = Standard Error

Table 6.2 (continued)

Group E (F = 0.12 ppm, n = 16)

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x μm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
	-19	39	-0.05	0.68	0.02	0.23
	18	36	-0.93	0.49	0.38	0.31
	78	71	0.25	0.70	0.54	0.49
	-9	32	0.56	0.59	-0.03	0.24
	46	22	0.84	0.74	0.22	0.18
	-22	26	0.45	0.55	-0.19	0.19
	-37	20	-0.71	0.42	-0.09	0.24
	15	44	-0.41	1.03	0.56	0.34
	-35	38	-0.77	1.13	0.13	0.29
	-8	28	0.20	0.50	-0.24	0.31
	-94	4	-0.10	0.23	0.05	0.01
	80	31	0.90	0.64	0.36	0.58
	48	73	1.00	0.74	0.44	0.51
	-9	65	0.83	0.75	0.11	0.37
	38	52	1.36	0.63	0.47	0.26
	-2	29	0.19	0.47	-0.17	0.40
mean	6	11	0.23	0.17	0.16	0.07

Group F (F = 0.5 ppm, n = 12)

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x μm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
	17	23	0.59	0.25	-0.52	0.15
	64	35	0.35	0.28	-0.36	0.30
	-29	36	0.95	0.55	-0.21	0.19
	35	42	1.53	0.66	0.00	0.36
	74	33	2.56	0.82	0.51	0.27
	133	104	0.30	1.06	0.74	0.35
	-68	139	-1.27	2.74	0.27	0.35
	4	71	0.41	0.88	0.34	0.38
	190	67	2.05	1.34	1.24	0.25
	1	47	0.90	0.37	-0.33	0.57
	88	39	-0.53	0.40	-0.54	0.78
	11	32	-0.01	0.28	-0.18	0.41
mean	27	23	0.65	0.31	0.08	0.16

* +ve values = remineralisation -ve values = demineralisation
SE = Standard Error

Table 6.3 Effect of low fluoride levels on Δz, surface zone (SZ), lesion body (LB) de- / remineralisation rates in a pH cycling study (3 hrs demin., 21 hrs remin. + F). Gelatin-prepared lesions

Group A (demineralisation only, n = 5)

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x µm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
	-1058	71	-1.69	0.55	-4.66	0.84
	-945	194	-0.48	0.47	-5.20	1.29
	-648	168	-0.54	0.09	-5.74	0.72
	-532	147	-1.30	0.43	-2.82	1.41
	-2347	213	-1.10	0.38	-2.10	0.35
mean	-1106	324	-1.02	0.23	-4.10	0.70

Group B (F < 0.03 ppm, n = 9)

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x µm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
	-34	63	0.18	0.64	-0.09	0.52
	-28	49	-0.12	0.33	-0.47	0.42
	-12	43	0.13	0.35	-0.12	0.38
	-115	55	-0.06	0.87	-0.34	0.36
	-140	184	-0.44	0.56	-0.42	0.77
	-127	205	0.22	0.78	0.34	0.87
	75	124	-0.50	0.46	-1.01	0.52
	-159	77	0.31	0.45	-0.18	0.42
	28	81	-0.10	0.25	-0.53	0.48
mean	-56	27	-0.04	0.10	-0.31	0.12

* +ve values = remineralisation
  -ve values = demineralisation
SE = Standard Error

Table 6.3 (continued)

Group C (F = 0.06 ppm, n = 10)

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x μm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
	-61	31	-0.20	0.04	-0.40	0.17
	-75	140	-0.08	0.52	-0.29	0.33
	-78	293	-0.37	1.26	-0.39	0.99
	29	26	0.84	0.35	-0.12	0.35
	-19	41	-0.12	0.54	-0.08	0.30
	16	30	0.57	0.11	-0.09	0.24
	-38	32	0.21	0.34	-0.40	0.25
	-44	54	-0.43	0.58	-0.29	0.33
	-5	29	-0.34	0.52	-0.01	0.11
	36	34	0.24	0.14	-0.03	0.24
mean	-24	13	0.03	0.13	-0.24	0.05

Group D (F = 0.09 ppm, n = 9)

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x μm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
	3	32	0.16	0.43	-0.15	0.40
	8	11	0.32	0.08	-0.08	0.17
	-13	71	0.59	0.42	-0.42	0.19
	-68	44	0.13	0.14	-0.59	0.28
	-58	45	0.00	0.57	-0.69	0.15
	-36	63	0.30	0.44	-0.70	0.22
	-36	21	-0.15	0.22	-0.38	0.26
	-41	30	0.11	0.17	-0.47	0.30
	6	33	0.00	0.33	-0.05	0.34
mean	-26	9	0.16	0.07	-0.39	0.08

* +ve values = remineralisation
-ve values = demineralisation
SE = Standard Error

Table 6.3 (continued)

Group E (F = 0.12 ppm, n = 11)

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x μm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
	35	65	-0.05	0.08	0.25	0.32
	95	50	0.06	0.30	0.25	0.27
	11	27	0.09	0.51	0.42	0.46
	36	11	0.96	0.12	0.42	0.08
	-4	16	0.29	0.27	0.04	0.31
	-66	36	-0.30	0.28	-0.24	0.24
	-67	56	0.09	0.13	-0.51	0.30
	-37	75	-0.48	0.53	-0.04	0.42
	50	67	0.59	0.44	0.13	0.43
	-179	36	-2.15	0.24	-0.78	0.29
	-80	58	-0.99	0.27	0.45	0.40
mean	-19	25	-0.17	0.25	0.05	0.12

Group F (F = 0.5 ppm, n = 6)

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x μm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
	-33	26	-0.52	0.33	-0.10	0.17
	54	51	0.39	0.37	0.32	0.31
	-41	98	-0.34	0.70	-0.29	0.61
	-78	62	-0.46	0.29	-0.55	0.37
	-36	147	1.28	0.70	-0.05	0.68
	122	82	0.05	0.28	0.56	0.34
mean	-2	31	-0.36	0.23	-0.02	0.16

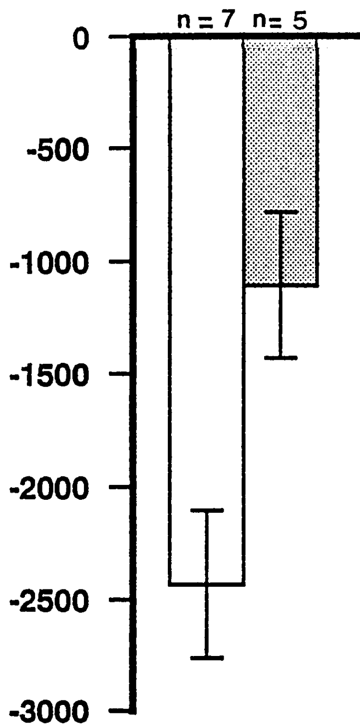
* +ve values = remineralisation
-ve values = demineralisation
SE = Standard Error

Group A (demineralisation only):

Demineralisation in the solution-prepared lesions was always greater than demineralisation in the gelatin-prepared lesions (Figure 6.1). The mean (± SE) Δz demineralisation rate for the solution-prepared lesions was -2433 (± 329) % vol. min. x µm / wk compared to -1106 (± 324) % vol. min. x µm / wk for the gelatin-prepared lesions. These values were statistically different ($0.01<p<0.02$, 2-tailed t-test). The mean surface zone demineralisation rate for the solution-prepared lesions -1.30 (± 0.45) % vol. min. / wk was not significantly different from the gelatin-prepared lesions -1.02 (± 0.23) % vol. min. / wk), whereas the mean lesion body demineralisation rate of the solution-prepared lesions -5.97 (± 0.48) % vol. min. / wk was statistically different $0.01<p<0.05$ from the corresponding value of the gelatin-prepared lesions -4.10 (± 0.70) % vol. min. / wk. ($0.01<p<0.05$).

pH cycled groups (Group B - Group F):

The mean Δz, surface zone and lesion body de- / remineralisation rates for both types of lesions of all groups are shown in Figures 6.2, 6.3 and 6.4. The mean Δz mineralisation rate of lesions increased with increasing fluoride concentration in the remineralising solution (Fig. 6.2). The Δz (± SE) demineralisation rates for the gelatin-prepared lesions decreased from -56 (± 27) % vol. min. x µm / wk with the 0.03 ppm fluoride remineralising solution to -2 (± 31) % vol. min. x µm / wk with the 0.5



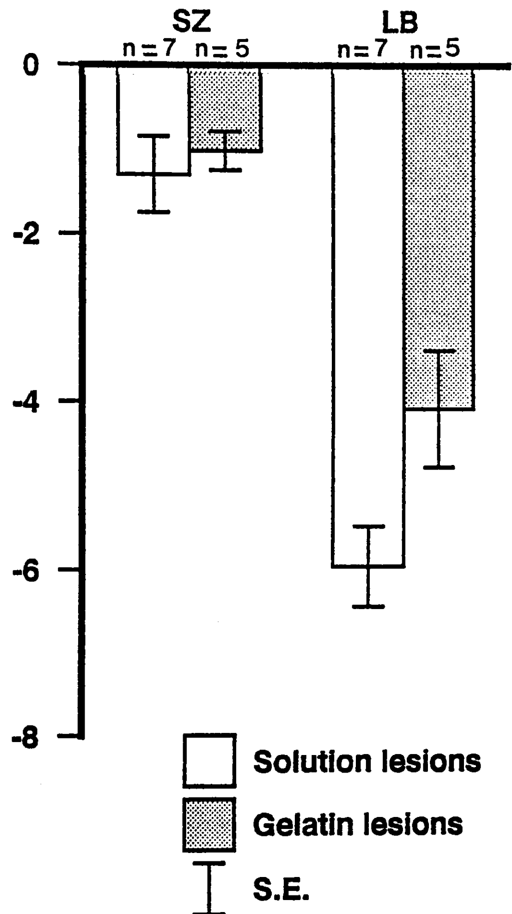


Figure 6.1 Mean Δz, surface zone (SZ) and lesion body (LB) mineralisation rates for the control group (A), subjected to continuous demineralisation

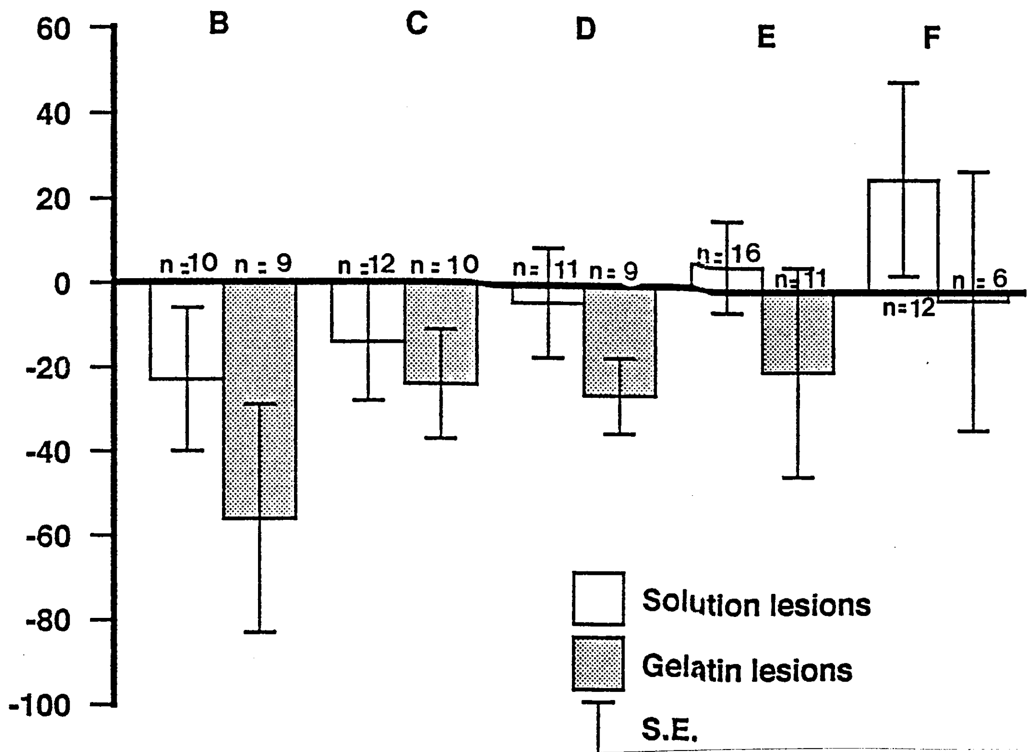


Figure 6.2 **Mean Δz mineralisation rates for the pH-cycled groups**

B - 0.03 ppm F in remineralising solution
C - 0.06 ppm F in remineralising solution
D - 0.09 ppm F in remineralising solution
E - 0.12 ppm F in remineralising solution
F - 0.50 ppm F in remineralising solution
+ve values = remineralisation
-ve values = demineralisation

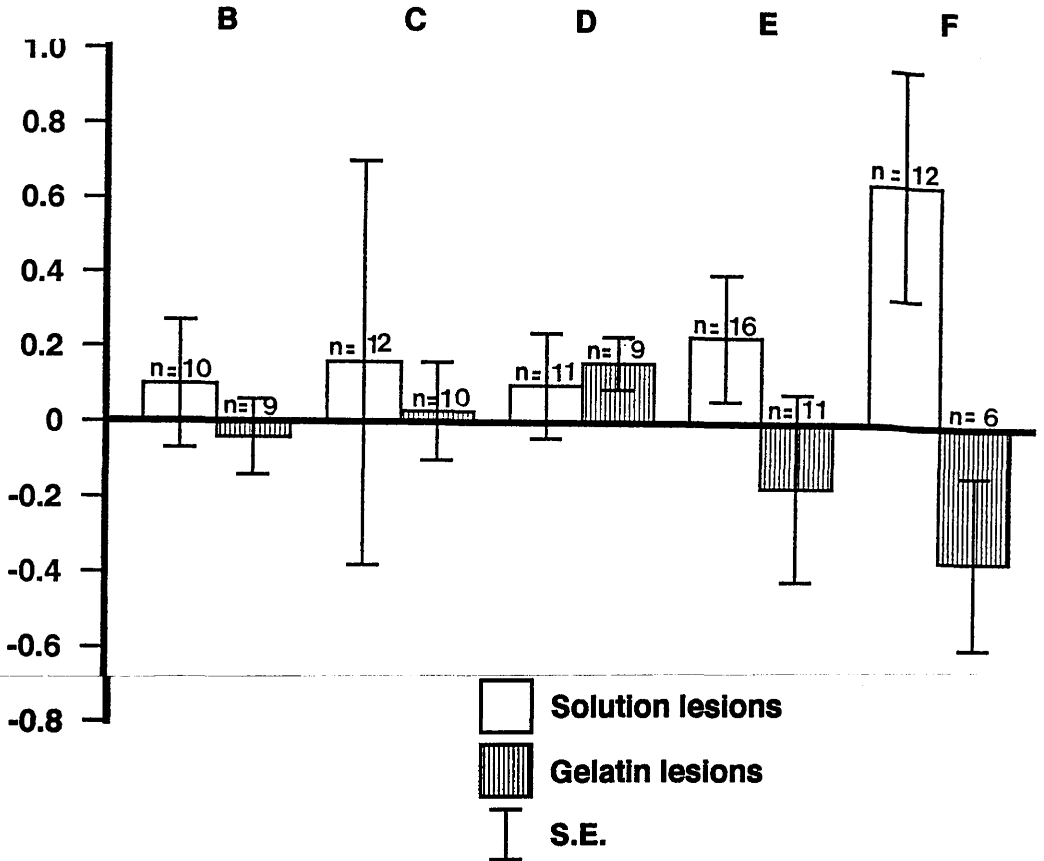


Figure 6.3 **Mean surface zone (SZ) mineralisation rates for the pH-cycled groups**
B - 0.03 ppm F in remineralising solution
C - 0.06 ppm F in remineralising solution
D - 0.09 ppm F in remineralising solution
E - 0.12 ppm F in remineralising solution
F - 0.50 ppm F in remineralising solution
+ve values = remineralisation
-ve values = demineralisation

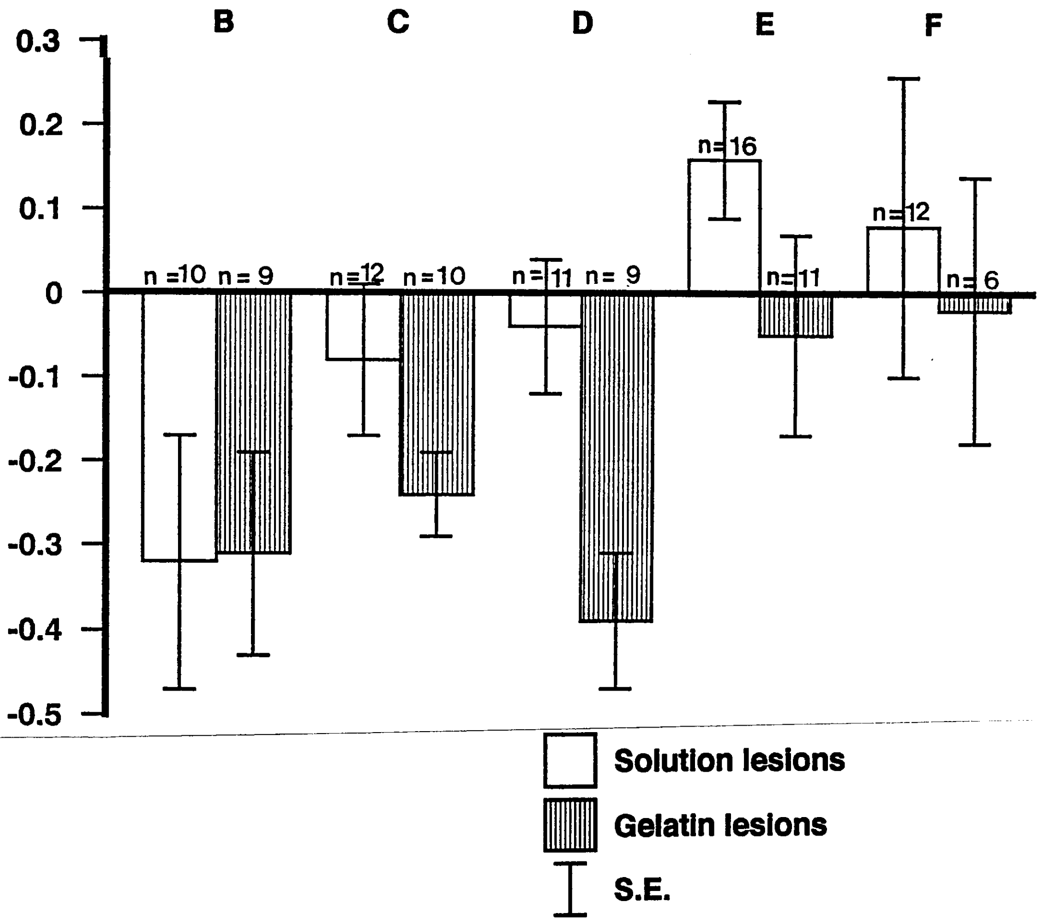


Figure 6.4 **Mean lesion body (LB) mineralisation rates for the pH-cycled groups**

B - 0.03 ppm F in remineralising solution
C - 0.06 ppm F in remineralising solution
D - 0.09 ppm F in remineralising solution
E - 0.12 ppm F in remineralising solution
F - 0.50 ppm F in remineralising solution
+ve values = remineralisation
-ve values = demineralisation

ppm fluoride remineralising solution. The solution-prepared lesions gave positive values when the fluoride concentrations in the remineralising solutions were 0.06, 0.12 and 0.5 ppm indicating net remineralisation. Analysis of variance for the full regression showed that, for the Δz parameter, both types of lesions showed a significant response to increase in fluoride concentration ($F = 3.77$, $p = 0.0004$). Further analysis for the order fitted showed a fluoride concentration effect ($F = 4.68$, $p = 0.0017$). Using a nested design (ie. lesion type nested with group) the effect of lesion type within each fluoride concentration was significant. ($F = 3.04$, $p = 0.0136$).

The mean values for the surface zone de- / remineralisation rates for the two types of lesions (Fig. 6.3), showed that surface zone remineralisation occurred for most groups, but only the solution-prepared lesions responded positively to an increase in fluoride concentration in the remineralising solution. Analysis of variance showed that for the solution-prepared lesions the effect of fluoride concentration was significant ($F = 5.083$, $p = 0.028$).

Again the mean lesion body mineralisation rate increased for both types of lesions. The mean (± SE) lesion body mineralisation rates ranged from -0.31 (± 0.12) % vol. min. / wk with the 0.03 ppm fluoride solution to -0.02 (± 0.16) % vol. min. / wk with the 0.5 ppm fluoride

solutions. The corresponding values for the solution-prepared lesions were -0.32 (± 0.15) % vol. min. / wk and 0.08 (± 0.16) % vol. min. / wk. These responses were, however, not statistically significant.

6.5 Discussion

The present study concluded that the continual presence of low levels of fluoride in a remineralising solution decreased the demineralisation rate of artificial lesions and more importantly enhanced the remineralisation of solution-prepared lesions with the magnitude of remineralisation being dependant on the fluoride concentration. Another pH-cycling study (Featherstone *et al.,* 1986) using sound enamel, also showed that mineral loss decreased progressively as the concentration of fluoride in the remineralising solution increased from 0.04 - 0.5 ppm. In addition dramatic enhancement of remineralisation was observed with the effect being dependant on the fluoride concentration. Such results show that the elevated fluoride concentrations between fluoride application, play a role in determining the balance between dissolution and precipitation.

Extrapolation to the *in vivo* situation is not easy because, *in vivo,* distribution of fluoride in saliva is complex (Weatherell *et al.,* 1984). The elevation of salivary fluoride concentration after frequent topical application normally lasts about 12 hours. The degree of fluoride elevation during this period depends on many

factors, including the fluoride concentration of the fluoride source; endogenous sources retained in and released from microbial and epithelial cells, and on the rate of oral fluoride clearance (Ekstrand, Lagerlof & Oliveby, 1986). Dawes (1983) showed that the rate of oral clearance of a solute depends most importantly on the volume of saliva before and after swallowing and on the salivary flow-rate. Such findings, together with the complexity of fluoride clearance at different sites in the mouth (Weatherell *et al.*, 1986) show that designing fluoride regimens to give a maximium therapeutic effect is difficult. Nonetheless, since it is not possible to establish clinically exactly when caries occurs, it is logical to recommend frequent exposure of enamel to agents containing low concentrations of fluoride (Joyston-Bechal & Kidd, 1982).

Finally, this study like several others (Mellberg & Chomicki, 1983; de Rooij & Nancollas, 1984; Damato, Strang & Stephen, 1988), showed that the method of artificial lesions preparation has important effect on the results of any subsequent de-/ remineralisation experiments. It is therefore concluded that solution-prepared lesions are more responsive, confirming the finding in the study reported in the previous chapter.

Chapter 7

Effect of daily five minute application of neutral sodium fluoride solutions - A pH cycling study.

7.1 Introduction

After a topical fluoride application (eg. fluoride rinsing or dentifrice usage), the salivary fluoride concentration reaches its maximium within seconds (Hassell, Gabathuler & Muhlemann, 1971; Stephen & Campbell, 1978; Oliveby *et al.,* 1989), this maximium being related to the concentration of the fluoride source (Bruun, Givskov & Thylstrup, 1984). The salivary fluoride clearance is multiexponential, with a rapid decrease, followed by other slower components and it is several hours before the basal fluoride concentration is regained (Ekstrand, Lagerlof & Oliveby, 1986). Fluoride elimination depends not only on the fluoride concentration of the source, but also on salivary flow rate and the redistribution into saliva of fluoride accummulated in the plaque and oral mucosa, (Oliveby *et al.,* 1989) presumbly in the form of calcium fluoride (Yao & Gron, 1970).

Controversy over the optimium fluoride doses remains unresolved and, to date, it is still not known to what extent the concentration of fluoride reflects on the caries preventive effect of a given solution. In an attempt to find the optimium concentration and length of

time for which the ionic fluoride concentration should be raised by exogenous fluoride, many workers have employed different fluoride vehicles, study sites and protocols. However, results from several studies are mixed. For instance, whereas several clinical trials support the conclusion that an increase in the fluoride concentration of a product will somewhat increase its anticaries effectiveness (Buhe, Buttner & Barlage, 1984; Lu *et al.*, 1987; Fogels *et al.*, 1988; Stephen *et al.*, 1988), others (Reed, 1973; Hodge *et al.*, 1980; Barlage, Buhe & Buttner, 1981; Koch *et al.*, 1982) have found conflicting results. Data from *in situ* studies are also mixed with a few studies (Goorhius & Purdell-Lewis, 1986; de Kloet *et al.*, 1986; Schafer, 1989) showing a significant increase in remineralisation of artificial lesions when the fluoride concentration in the product was increased. *In vitro* work (Mellberg & Mallon, 1984; ten Cate & Simmons, 1988; Featherstone, Shariati & Brugler, 1988) showed that an optimium fluoride dose exists beyond which the efficacy of fluoride is not increased.

In this chapter, the pH cycling model was used to simulate the high fluoride concentrations present immediately after a topical fluoride application so that quantitative information regarding optimium fluoride doses could be obtained. Such conditions were achieved by exposing enamel sections daily, for five minutes, to neutral sodium fluoride solutions while ensuring that no

carry over of fluoride solution took place. Neutral sodium fluoride solutions were chosen because they have a simple and straightforward formulation. The mineral content changes in enamel specimens were measured and related to the fluoride content in the various solutions employed. The concentrations of fluoride in the solutions were 0, 1 250, 500 and 1000 ppm fluoride.

In view of the results obtained, (Section 7.3), namely a non-significant increase in remineralisation between the 500 and 1000 ppm F groups, a second experiment was carried out to include sodium fluoride solutions of higher fluoride concentrations (1750 and 2500 ppm fluoride).

7.2 Materials and methods

This section gives details of the materials and methods used for the two experiments.

Experiment 1

Artificial carious lesions were prepared on the buccal surfaces of 25 human premolar teeth using the buffer system (Section 2.2.3). Thereafter enamel sections were prepared and randomly allocated to six groups, each group containing ten sections, with a maximum of four lesions on each section. Specimens were subjected to pH cycling, employing conditions described in Chapter 6. However, between the demineralising and remineralising phases, specimens were also immersed daily, for five minutes, into

neutral sodium fluoride solutions. After the five minute treatment, specimens were dabbed dry, rinsed in artificial saliva and stored in fresh artificial saliva for the remainder of the day. This procedure was necessary to avoid carry-over of sodium fluoride solution. Control sections (Group A) were rinsed in double-distilled de-ionised water for five minutes per day, whereas sections in Groups B, C, D, and E were immersed daily for five minutes in neutral sodium fluoride solutions containing 1, 250, 500 and 1000 ppm fluoride. Treatment with fluoride solution was always carried out immediately after the demineralisation period although it has been shown (ten Cate *et al.*, 1988) that lesion progression will be observed, irrespective of the timing of the fluoride therapy. The demineralising solution and artificial saliva were regularly analysed for fluoride, but levels were always found to be below 0.03 ppm. Specimens were cycled in this manner daily for five weeks. During this time, microradiographs of the sections were taken once per week. At the end of the experiment, sections from each group were imbibed in water and quinoline and examined with polarized light.

Experiment 2

The experiment described above was repeated, only on this occasion 20 teeth were used. In this case specimens were exposed for five minutes to rinsing solutions containing 0 ppm fluoride (Group A1), 1750 ppm fluoride (Group F) and 2500 ppm fluoride (Group G). Polarising microscopy

was also carried out on these specimens.

7.3 Results

The de-/ remineralisation rate values of lesions in all groups (ie A - G) are tabulated in Table 7.1. The number of microradiographic tracings used in this study was 852 because only lesions exhibiting a narrow range of Δz values(between 2000 and 4000 % vol. min. x µm at the beginning of the experiment (Table 7.2) were used in the analysis. In this way any effect of lesion size on remineralisation was minimised (Strang *et al.,* 1987)

Experiment 1

The mean de-/ remineralisation rates for the Δz, surface zone (SZ), and lesion body (LB) parameters of lesions in the five groups are illustrated in Figures 7.1 - 7.3. The mean Δz ($\pm$ SE) mineralisation rates (Fig. 7.1) of lesions in Groups A and B were -74 ($\pm$ 23) and -85 ($\pm$ 48) % vol. min. x µm / wk respectively. The surface zone (Fig. 7.2) and lesion body (Fig. 7.3) parameters of Groups A and B lesions also demineralised, as did the sound enamel adjacent to the lesions in these two groups. There were no significant differences between these two groups. However, Groups A and B were significantly different from all other groups ($p < 0.05$, 1-tailed t-test). In the other Groups (C - E), net remineralisation occurred for all three parameters, although seven of the 29 lesions analysed in Group C and four of the 23 lesions analysed in Group D demineralised. The mean Δz

Table 7.1 Effect of daily fluoride exposure on Δz, surface zone (SZ), lesion body (LB) de- / remineralisation rates. A pH cycling study

Experiment 1, Group A: (F < 0.02 ppm) n = 15

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x μm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
	-29	69	-3.94	0.50	0.02	0.34
	-117	114	-1.67	1.10	-1.08	1.12
	25	84	-1.10	1.39	-0.67	1.24
	-35	47	-0.72	0.95	-0.20	0.95
	-44	93	-0.66	0.75	-0.79	1.06
	-80	70	-0.91	1.15	-1.96	0.70
	44	133	1.17	1.21	0.48	0.74
	-64	34	-1.01	0.70	-1.11	0.55
	-186	66	-1.70	0.75	-2.28	0.62
	100	121	0.80	0.85	0.32	1.10
	-200	72	-1.07	1.26	-2.20	0.84
	-93	93	-0.39	0.98	-1.04	0.57
	-96	71	-0.60	0.97	-0.46	0.67
	-127	110	-1.46	0.86	-1.03	1.19
	-204	157	-2.93	2.54	-2.85	2.15
mean	-74	24	1.08	0.32	-0.95	0.27

Experiment 1, Group B: (F = 1 ppm) n = 11

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x μm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
	71	55	-0.68	0.87	-0.36	0.81
	-49	59	0.65	0.77	-1.03	0.73
	-196	81	0.33	1.21	0.02	1.29
	-36	66	0.21	0.72	-1.72	0.90
	-68	26	0.42	0.56	-2.05	0.76
	194	114	2.29	1.49	-1.43	0.68
	-278	188	-0.89	1.10	-2.45	0.87
	-343	95	-2.31	1.06	-3.20	0.78
	23	32	0.64	0.32	0.15	0.70
	-224	47	-2.28	0.59	-0.54	0.65
	-30	285	-0.16	0.18	0.55	1.81
mean	-85	48	-0.16	0.40	-1.10	0.36

* +ve values = remineralisation -ve values = demineralisation
SE = Standard Error

Table 7.1 (continued)

Experiment 1, Group C: (F = 250 ppm) n = 29

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x μm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
	97	89	2.22	0.75	1.01	0.40
	19	61	1.03	0.59	1.06	0.59
	103	58	1.93	1.02	2.31	0.75
	14	101	0.70	1.33	0.12	1.29
	77	32	1.81	0.92	0.50	0.58
	181	25	2.03	0.48	1.29	0.46
	89	35	0.24	0.54	1.11	0.59
	61	76	0.79	0.91	0.56	0.79
	-39	43	-0.59	0.77	0.01	1.13
	12	73	0.35	1.10	0.11	0.48
	79	56	1.07	0.89	0.98	0.49
	-92	99	-0.07	1.17	0.30	0.80
	54	44	0.86	0.54	0.84	0.34
	287	68	1.19	1.76	3.75	0.60
	48	57	0.72	0.81	0.84	0.72
	-15	12	-0.73	0.60	0.39	0.29
	86	93	0.43	1.18	1.88	0.96
	51	77	0.65	1.05	0.29	0.57
	-21	49	0.01	0.59	0.07	0.63
	40	31	1.01	0.57	0.60	0.28
	76	35	1.52	0.43	0.53	0.39
	84	71	2.20	1.13	2.11	1.10
	100	31	1.66	0.37	1.47	0.60
	176	89	-1.58	0.97	1.62	0.83
	-147	67	-1.13	0.78	-0.32	0.62
	109	116	1.42	1.05	1.22	1.95
	64	77	0.65	0.99	0.82	0.92
	-177	63	-0.67	0.51	-2.39	0.84
	327	142	1.55	0.53	1.46	1.13
mean	**60**	**19.6**	**0.73**	**0.19**	**0.84**	**0.19**

* +ve values = remineralisation
-ve values = demineralisation
SE = Standard Error

Table 7.1 (continued)

Experiment 1, Group D: (F = 500 ppm) n = 23

	De- / Remineralisation Rates*					
	Δz (% vol. min. x μm / wk)	SE	SZ (% vol. min. / wk)	SE	LB (% vol. min. /wk)	SE
	575	102	5.80	1.02	6.31	0.99
	173	34	2.80	0.53	1.77	0.33
	101	21	2.00	0.48	1.32	0.49
	270	44	1.83	0.85	1.98	0.85
	22	107	0.17	1.12	-0.01	1.06
	177	136	2.79	0.88	2.54	0.34
	-111	256	3.47	4.07	4.62	3.16
	72	47	-3.21	0.61	2.50	0.91
	369	89	3.57	0.70	4.65	0.52
	-19	60	0.54	0.65	0.65	0.61
	145	33	0.59	0.41	2.08	0.46
	56	37	1.17	0.59	0.85	0.64
	38	45	0.17	0.27	1.20	0.44
	177	85	2.72	0.85	2.44	0.60
	-1	37	-0.94	0.53	1.71	0.49
	-35	88	-0.03	1.08	1.18	0.59
	5	39	0.27	0.33	0.62	0.55
	126	48	1.15	0.30	2.03	0.38
	180	53	0.58	0.61	1.41	0.54
	311	64	3.42	0.35	2.30	0.24
	243	55	2.07	0.47	3.89	0.59
	159	64	1.84	0.72	1.96	0.61
	-11	98	0.40	0.78	0.87	0.98
mean	131	36	1.44	0.39	2.12	0.32

* +ve values = remineralisation
-ve values = demineralisation
SE = Standard Error

Table 7.1 (continued)

Experiment 1, Group E: (F = 1000 ppm) n = 24

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x µm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
	14	36	-0.45	0.66	0.58	0.63
	9	17	-0.10	0.30	0.56	0.22
	56	26	-0.06	0.67	1.06	0.29
	194	34	2.14	0.60	3.29	0.58
	70	42	1.21	0.49	1.08	0.39
	202	118	1.10	1.48	2.43	1.13
	21	155	-0.97	1.86	1.71	1.52
	146	7	0.76	0.43	1.73	0.32
	373	76	2.97	1.08	5.42	1.01
	42	45	0.43	0.41	2.12	0.51
	174	49	-1.11	1.19	3.02	0.89
	437	110	4.60	1.44	6.02	1.11
	382	96	2.91	1.05	5.01	0.88
	446	43	1.87	0.62	6.45	0.31
	145	9	0.35	0.24	1.67	0.28
	147	67	1.41	0.86	1.74	1.06
	271	27	2.70	0.69	3.10	0.44
	288	93	1.98	0.94	2.28	0.78
	241	124	1.27	1.27	3.09	1.44
	83	65	0.78	1.10	1.76	0.48
	161	23	0.90	0.58	3.18	0.71
	406	132	3.93	0.72	5.97	1.15
	198	82	0.82	1.12	3.17	1.15
	34	51	0.78	0.79	0.53	0.63
mean	189	29	1.26	0.29	2.79	0.37

* +ve values = remineralisation
-ve values = demineralisation
SE = Standard Error

Table 7.1 (continued)

Experiment 2, Group A1: (F < 0.02 ppm) n = 5

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x μm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
	-25	32	-0.66	0.33	0.17	0.42
	-119	59	-1.26	0.39	-0.58	0.47
	34	79	0.14	0.27	-0.92	0.40
	-81	11	-0.48	0.41	-0.71	0.25
	-11	48	-0.27	0.76	0.47	1.19
mean	-40	27	-0.51	0.23	-0.31	0.27

Experiment 2, Group F: (F = 1750 ppm) n = 16

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x μm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
	186	41	1.13	0.48	2.79	0.71
	218	47	1.57	0.68	3.48	0.74
	176	30	1.79	0.20	2.69	0.46
	130	25	0.73	0.65	2.36	0.29
	207	47	3.15	0.79	3.22	0.59
	255	43	3.31	0.56	3.19	0.43
	167	59	1.22	0.66	2.46	0.98
	164	31	2.30	0.69	3.07	0.45
	247	61	2.50	0.95	3.67	0.46
	149	39	1.64	0.70	2.06	0.34
	109	35	1.21	0.66	2.45	0.24
	61	38	0.46	0.77	1.95	0.82
	218	46	0.43	0.89	3.55	0.26
	177	80	0.88	1.08	3.23	0.74
	184	14	0.95	0.46	2.84	0.13
	103	40	0.93	0.53	2.27	0.70
mean	172	13	1.51	0.22	2.83	0.13

* +ve values = remineralisation
-ve values = demineralisation
SE = Standard Error

Table 7.1 (continued)

Experiment 2, Group G: (F = 2500 ppm) n = 11

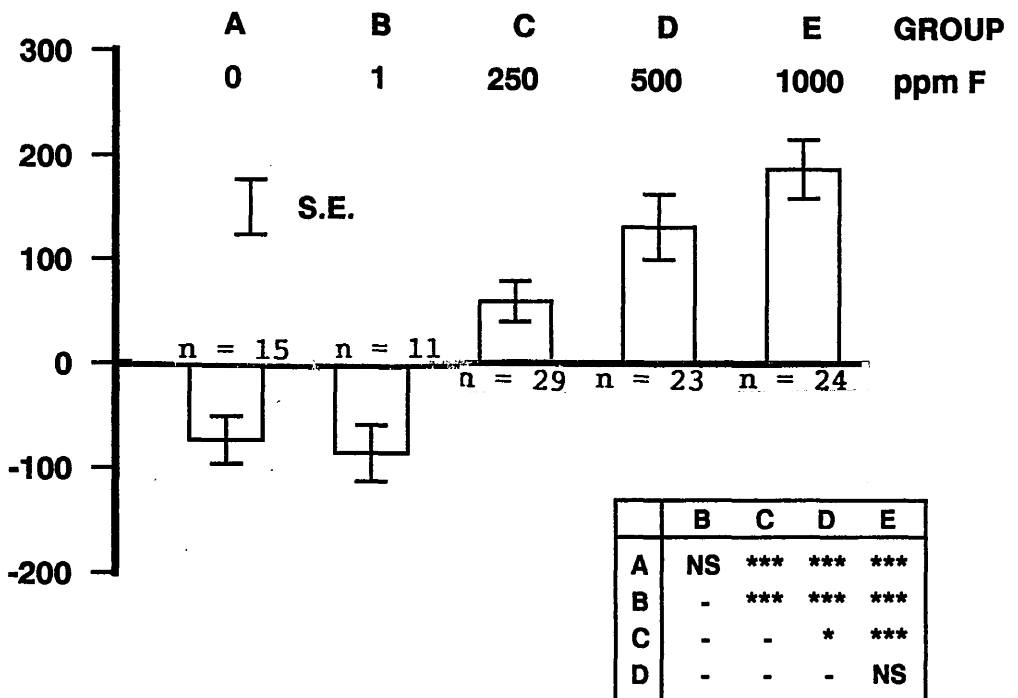
	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x μm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
	132	37	0.81	0.53	1.65	0.51
	177	78	-0.09	0.97	3.13	0.64
	265	111	0.82	1.28	4.10	0.98
	105	31	-0.29	0.65	2.79	0.69
	175	52	1.15	0.76	3.43	1.09
	333	107	2.18	1.25	4.60	1.01
	317	84	2.35	1.00	3.52	0.68
	255	62	-1.26	0.66	4.09	0.65
	205	33	-0.17	0.55	3.29	0.70
	131	37	1.55	0.65	2.08	0.60
	45	114	-0.22	1.25	0.50	1.14
mean	195	27	0.62	0.34	3.02	0 36

* +ve values = remineralisation
-ve values = demineralisation
SE = Standard Error

Table 7.2 Mean (SE) baseline values for Δz, surface zone (SZ) and lesion body (LB) for the different experimental groups

	Mean baseline values						
	Δz (% vol. min. x μm)	SE	SZ (% vol. min.)	SE	LB (% vol. min.)	SE	n
A	3621	280	55.5	1.5	43.0	2.3	20
B	3131	228	58.2	1.4	44.5	1.8	11
C	3000	187	60.4	1.2	47.8	1.8	29
D	3178	224	58.1	1.8	44.3	1.9	23
E	2951	241	60.7	1.9	45.1	2.5	24
F	2760	139	56.6	1.5	46.5	1.9	16
G	3193	176	58.8	1.5	38.8	1.5	11

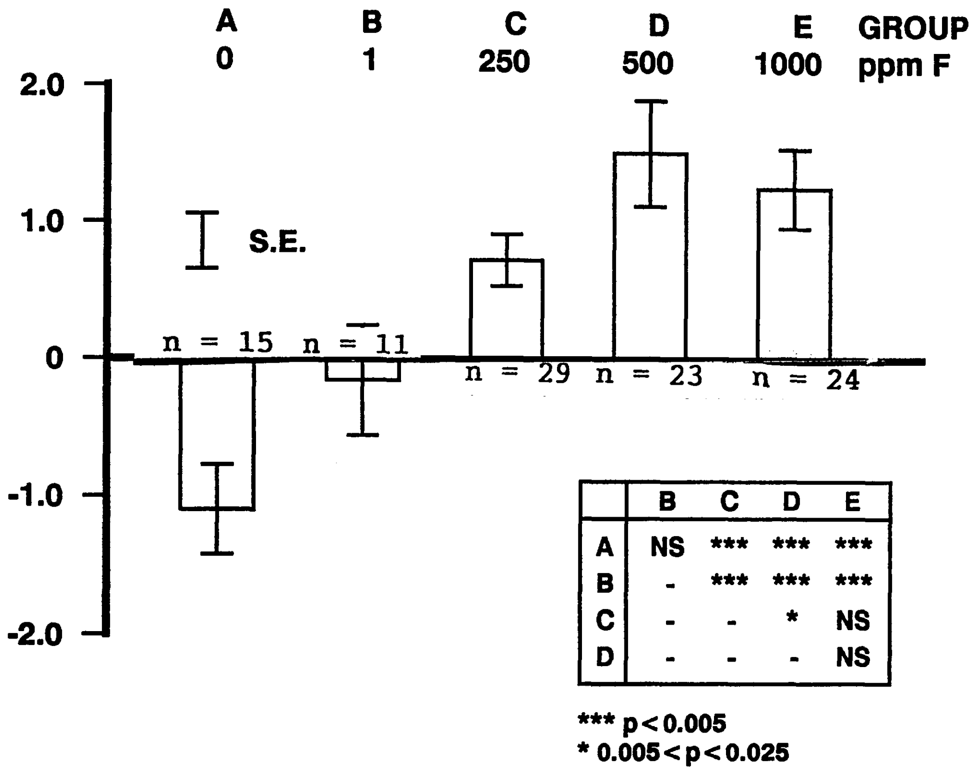
Δz Mineralisation Rate
(% vol. min. x μm / wk)



	B	C	D	E
A	NS	***	***	***
B	-	***	***	***
C	-	-	*	***
D	-	-	-	NS

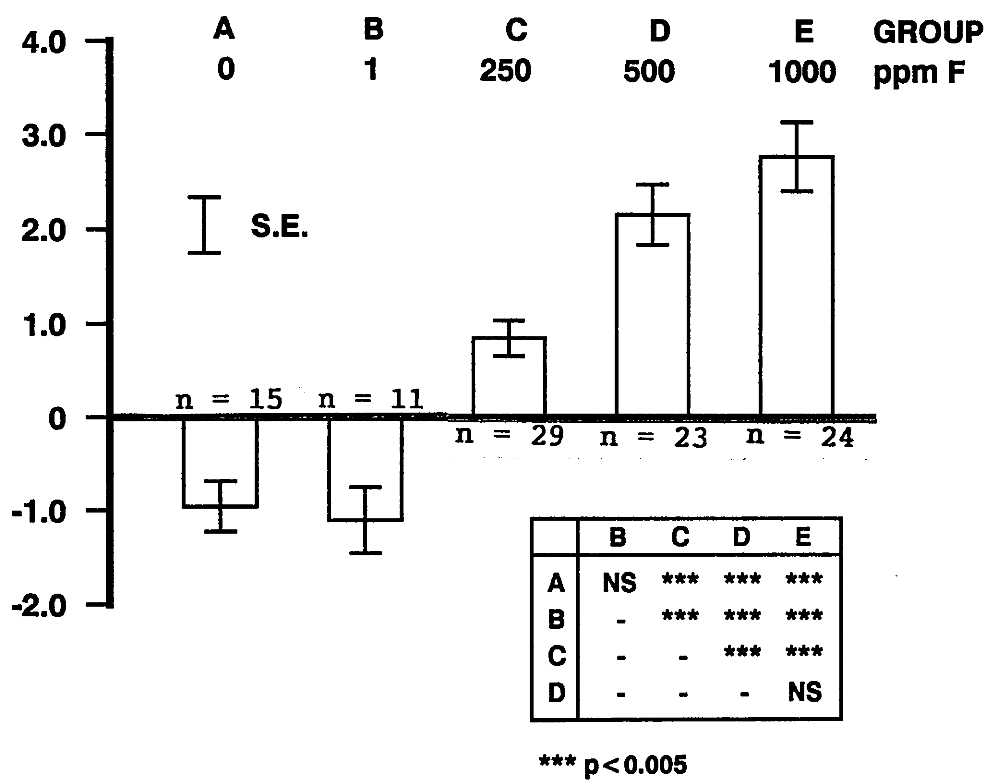
*** $p < 0.005$
* $0.005 < p < 0.025$

Figure 7.1 **Mean z mineralisation rates for lesions in Group A - E (Experiment 1)**
+ve values = remineralisation
-ve values = demineralisation



	B	C	D	E
A	NS	***	***	***
B	-	***	***	***
C	-	-	*	NS
D	-	-	-	NS

Figure 7.2 **Mean surface zone (SZ) mineralisation rates for lesions in Groups A - E (Experiment 1)**
+ve values = remineralisation
-ve values = demineralisation



	B	C	D	E
A	NS	***	***	***
B	-	***	***	***
C	-	-	***	***
D	-	-	-	NS

*** $p < 0.005$

Figure 7.3 **Mean lesion body (LB) mineralisation rates for lesions in Groups A - E (Experiment 1)**
+ve values = remineralisation
-ve values = demineralisation

mineralisation rate for Group C (60 ± 20 % vol. min. x μm / wk) was significantly lower than the corresponding values for Group D (132 ± 32 % vol. min. x μm / wk, $0.01 < p < 0.05$) and Group E, (189 ± 29 % vol. min. x μm / wk, $p < 0.005$). The mean lesion body and surface zone mineralisation rates showed similar trends to the Δz values. No significant differences were obtained for lesions treated with 500 ppm and 1000 ppm fluoride.

Experiment 2

The mean Δz, surface zone (SZ) and lesion body (LB) mineralisation rate values of Groups A1, F and G are illustrated in Figure 7.4. Group A1 was a repeat of the control group with lesions exposed daily to double-distilled deionised water for five minutes. For all parameters, Group A1 was statistically different from the fluoride treated groups ($p < 0.005$). The mean Δz mineralisation rate values of Group F, 172 (± 13) % vol. min. x μm / wk was not significantly different from the mean Δz mineralisation rate value of Group G, 195 (± 27) % vol. min. x μm / wk. Similarly there was no significant difference between the mean lesion body mineralisation rate values of Group F and Group G, although the surface zone mineralisation rate of Group G was unexpectedely low and was significantly different from that of Group F ($0.01 < p < 0.05$).

Since there were no significant differences between the control groups of the two experiments (Group A and Group

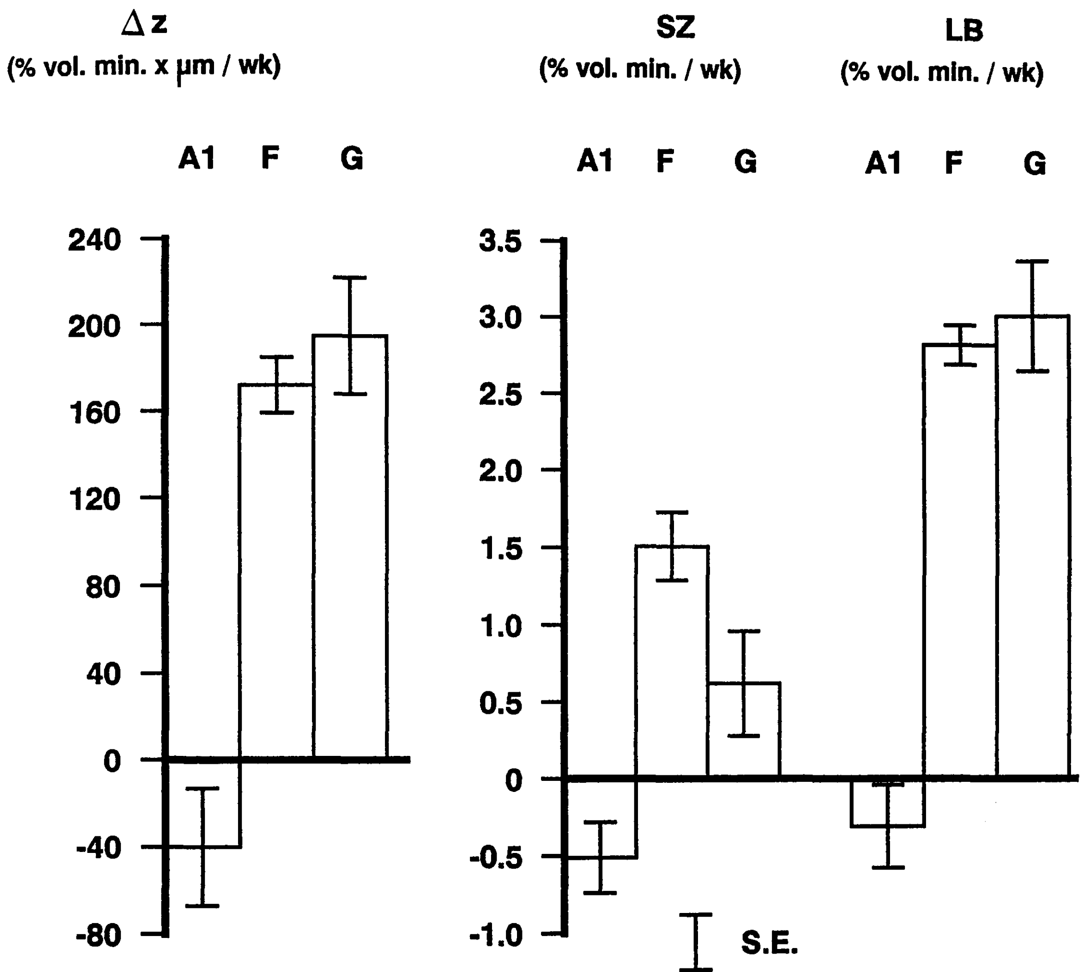


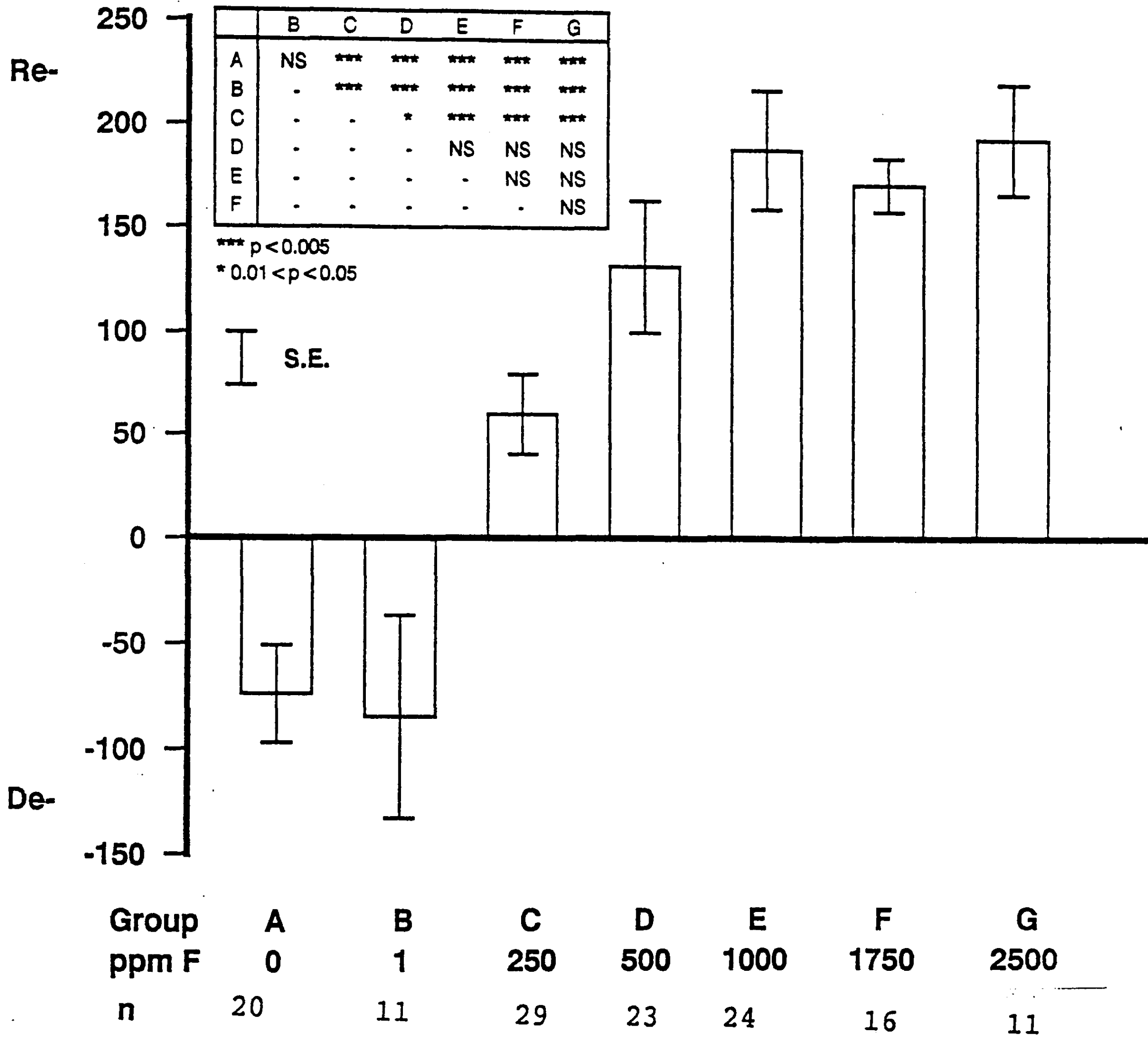
Figure 7.4 Mean Δz, surface zone (SZ) and lesion body (LB) mineralisation rates for lesions in Groups A1 (control), F (1750 ppm F) and G (2500 ppm F) in rinsing solution. (Experiment 2)
+ve values = remineralisation
-ve values = demineralisation

A1), the data of all groups were combined for all three parameters (Fig. 7.5 - Fig. 7.7). Results show that there were no significant differences between the Δz, surface zone, and lesion body mineralisation rate values of Groups D - G.

The polarising photomicrographs of samples belonging to Groups A, A1 and B showed typical zones when imbibed in water and quinoline. Most lesions in Group C (250 ppm F) and a few lesions in Group D (500 ppm F) showed laminations (Fig. 7.8) when imbibed in water and lesions in Group E (1000 ppm F), when imbibed in quinoline showed a broadening of the dark zone (Fig. 7.9).

7.4 Discussion

This study showed that a daily, five minute application of neutral sodium fluoride solution effectively enhanced the remineralisation of artificial enamel lesions. No significant differences in remineralisation were found when the fluoride concentration was increased above 500 ppm, indicating that under these conditions the optimium fluoride concentration was reached. Other studies (Mellberg & Mallon, 1984; ten Cate & Simmons, 1986; Featherstone, Shariati & Brugler, 1988) recorded different optimium fluoride concentrations. However direct comparison between these studies is not possible because different experimental protocols and fluoride vehicles were employed. Caution must be exercised when comparing different fluoride vehicles



	B	C	D	E	F	G
A	NS	***	***	***	***	***
B	-	***	***	***	***	***
C	-	-	*	***	***	***
D	-	-	-	NS	NS	NS
E	-	-	-	-	NS	NS
F	-	-	-	-	-	NS

Figure 7.5 **Mean Δz mineralisation rates for lesions in all groups (Experiment 1 and 2)**
+ve values = remineralisation
-ve values = demineralisation

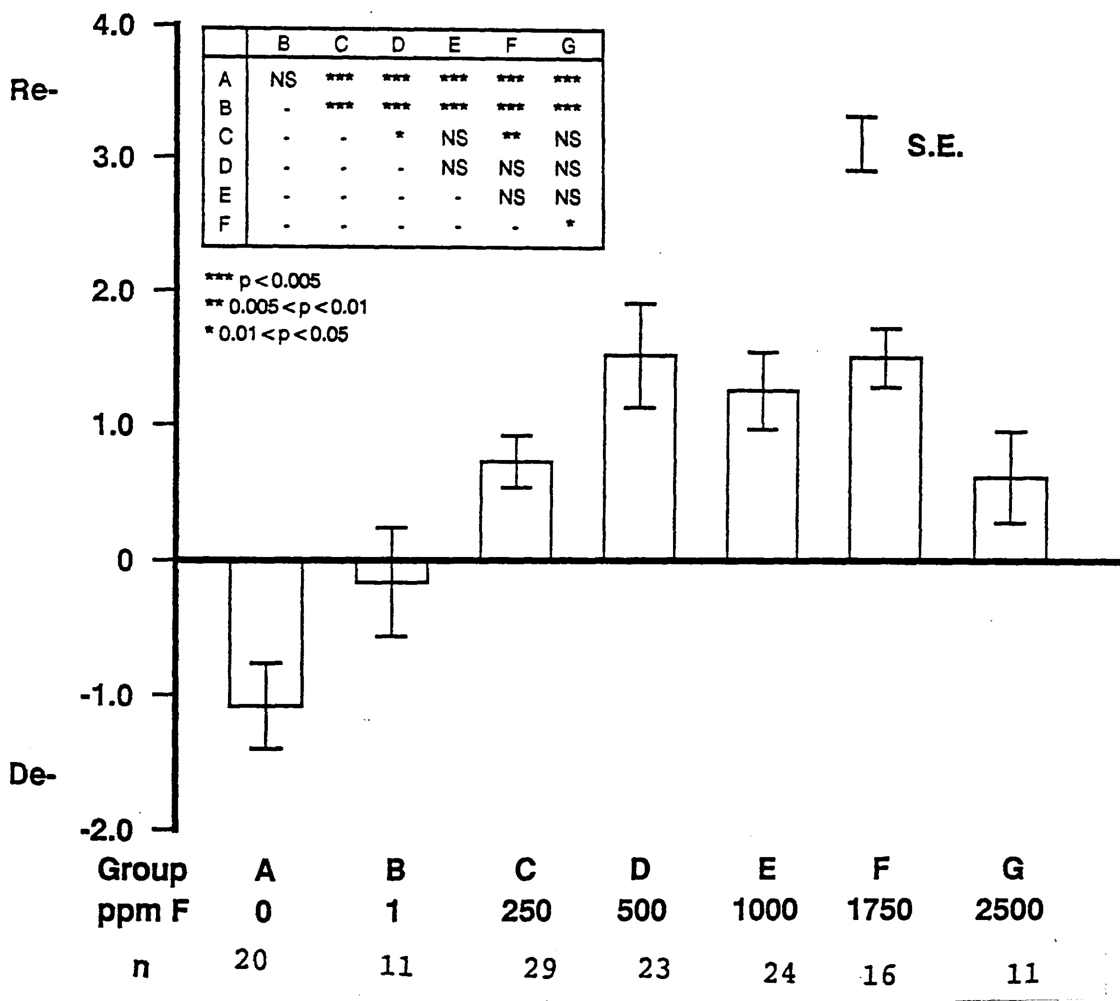


Figure 7.6 **Mean surface zone (SZ) mineralisation rates for lesions in all groups (Experiments 1 and 2)**
+ve values = remineralisation
-ve values = demineralisation

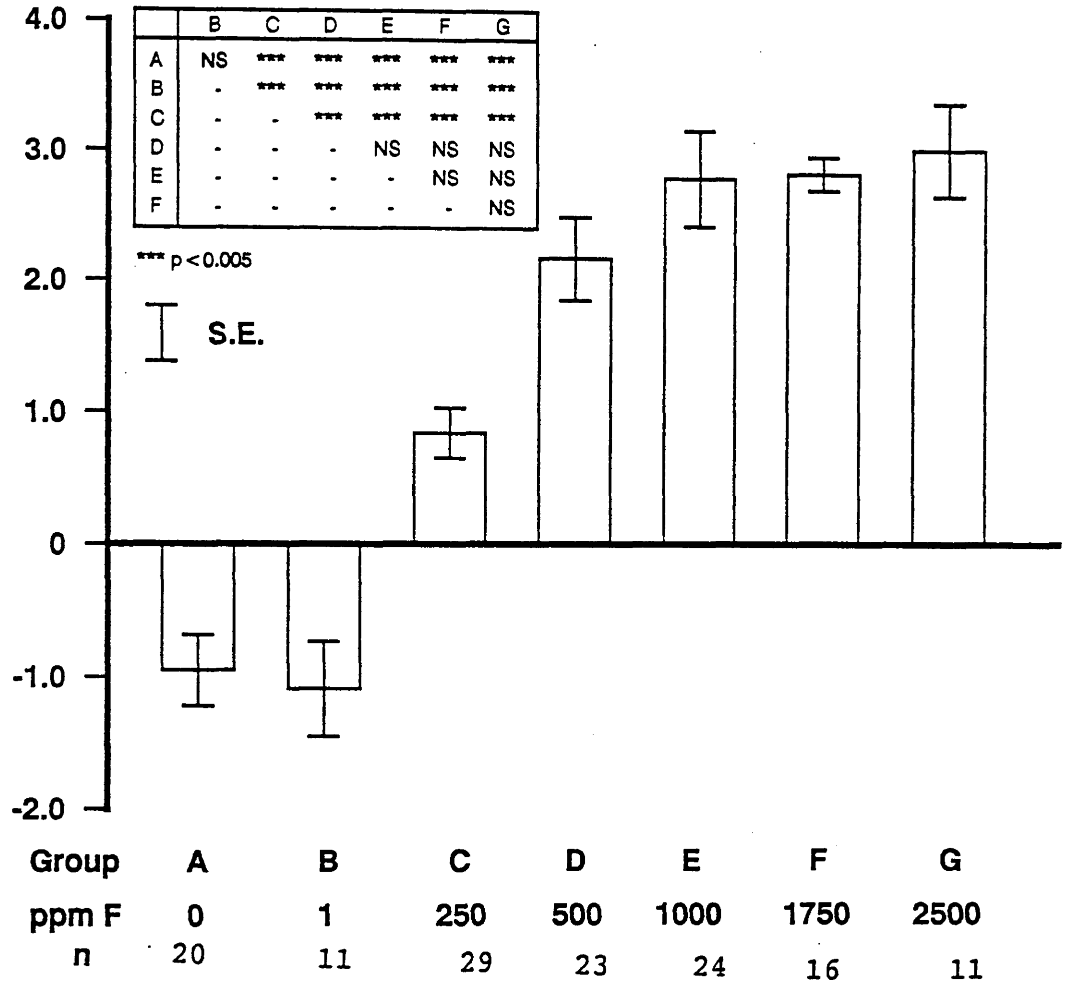


Figure 7.7 **Mean lesion body (LB) mineralisation rates for lesions in all groups (Experiment 1 and 2)**

+ve values = remineralisation
-ve values = demineralisation

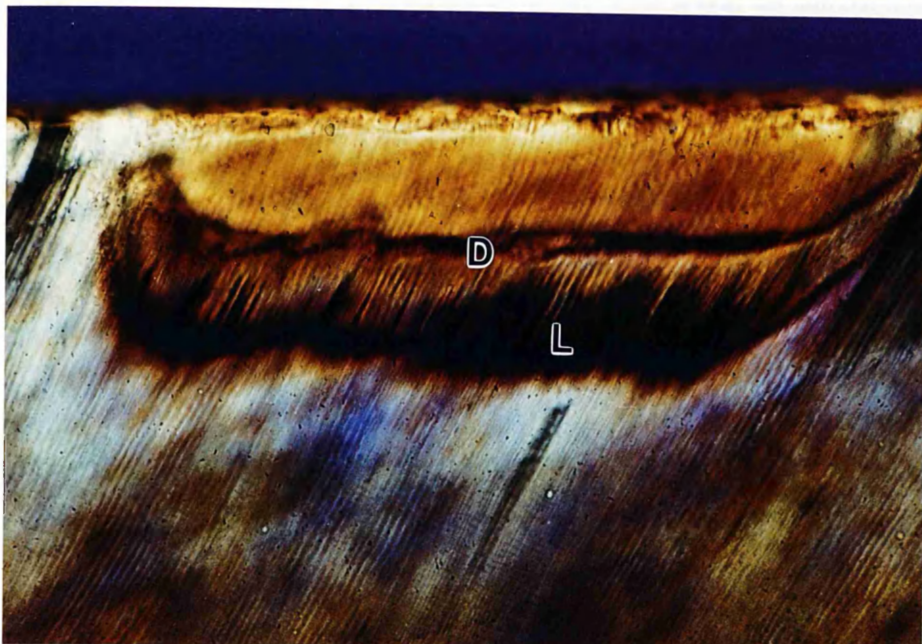


Figure 7.8 Photomicrograph of a laminated lesion from Group D (250 ppm F), examined in quinoline with polarized light, after five weeks pH-cycling (D = dark zone, L = lamination)

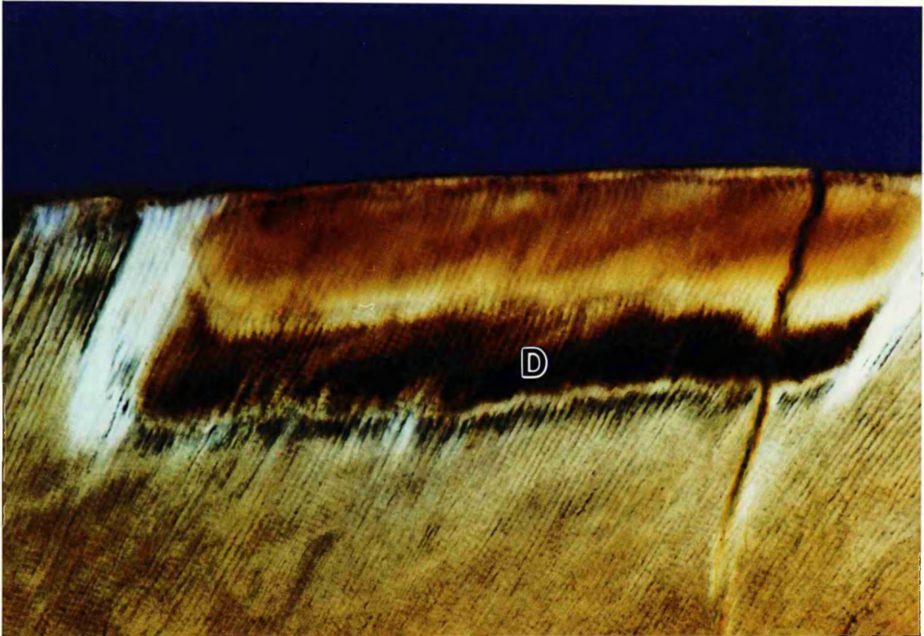


Figure 7.9 Photomicrograph of a lesion from Group E (500 ppm F), examined in quinoline with polarized light, after five weeks pH-cycling (D = broadened dark zone)

because, although the fluoride concentration in a particular topical solution might be higher than in an other, both the free fluoride ion concentration and its biological activity may be lower in the former solution due, for instance, to the formation of ion pairs (Larsen & Jensen, 1986).

The elevated free fluoride concentration immediately after brushing is of crucial importance in decreasing the susceptibility of enamel to caries (ten Cate & Simmons, 1986) presumbly by forming calcium fluoride-like material. Chemical analysis, Raman spectroscopy and scanning electron microscopy have all comfirmed the presence of calcium fluoride inside lesions *in vivo.* Several other studies (Geroud, 1945; Leach, 1959; Caslavska, Moreno & Brudevold, 1975; Grobler, Ogaard & Rolla, 1981; Ogaard, Rolla & Helgeland, 1983a; 1983b; Arends, Reinstema & Dijkman, 1988; Saxegaard & Rolla, 1988) have demonstrated the presence of calcium fluoride-like material in lesions, and on sound enamel, for prolonged periods after topical applicaton of various fluoride agents. Despite this, the role of calcium fluoride in preventing caries is still highly controversial. For some time it was assumed that calcium fluoride is rapidly lost from the oral cavity (McCann, 1968; Brudevold *et al.,* 1967) and therefore its formation was of no significance for the cariostatic potential of fluoride. Recent research (Kanaya, *et al.,* 1983; Rolla & Ogaard, 1986) has shown that calcium

fluoride, when present in the oral cavity, has unique properties which make it less soluble than when present in water, due to a mechanism involving phosphate ions (Kanaya *et al.*, 1983). When the pH is high the calcium fluoride-like material remains as insoluble globules, which are not beneficial (Arends, Reinstema & Dijkman, 1988). However, with large changes in pH slow dissolution of the globules takes place, releasing fluoride ions and therby promoting remineralisation (Mellberg, 1977; Larsen *et al.*, 1981; Fejerskov, Thylstrup & Larsen, 1981). The formation of calcium fluoride depends on the pH, on the concentration of fluoride in the topical solution and on the exposure time (Larsen & Jensen, 1986). However, caries preventive measures always involve so much fluoride that one assumes that this compound is formed. In addition an *in vitro* study (Duschner, Uchtmann & Ahrens, 1989) showed that calcium fluoride is also formed with neutral solutions. In the present experiment, extraction of alkali soluble fluoride (Caslavska, Moreno & Brudevold, 1975) was not possible due to the small size of the enamel specimens, however from the above discussion, the presence of calcium fluoride cannot be excluded. In fact it is highly likely that the cariostatic effect observed from the daily five minute fluoride rinse involved the formation of loosely bound calcium fluoride like material.

The polarising photomicrographs support the microdensitometric results. For instance the laminations

observed in most Group C lesions were due to incomplete or non-continuous remineralisation (Sato & Yamamoto, 1986). The absence of laminations, together with the observed broadened dark zones in Groups E - G indicate the efficiency of the remineralisation process (Silverstone & Poole, 1968) or as Crabb (1966) suggested, the presence of arrested lesions. This could explain why lesion remineralisation was not affected by fluoride concentrations above 500 ppm fluoride.

In conclusion, this study although representing a simple situation when compared to conditions *in vivo*, shows that the optimium fluoride concentration reached immediately after fluoride application is important in providing maximium cariosatic protection.

Chapter 8

In situ de-/ remineralisation studies

8.1 Introduction

As described in Section 1.6.6, *in situ* studies provide a natural environment for de-/ remineralisation processes to take place and, since the specimens can be removed, they allow quantification of lesion mineral content. *In situ* studies, therefore, can provide potential information regarding the benefits of anticaries systems and, at the same time, might decrease the need for lengthy, high cost, clinical trials. The *in situ* model used in this study had an added advantage over other models in that the enamel specimens consisted of thin sections. As discussed in Section 1.6.3, the use of thin sections allows repeat measurements of mineral content of the same area of enamel and therefore permit more accurate quantification of mineral content.

Several clinical studies (Buhe, Buttner & Barlage, 1984; Lu *et al.,* 1987; Fogels *et al.,* 1988) have shown that the anticaries activity of fluoride is increased with increasing concentration of fluoride in the agent, although such findings have not always been statistically significant. In a recent clinical trial, a significant relationship between fluoride concentration in sodium monofluorophoshate (SMFP) dentifrices and DMFS

increments was obtained (Stephen *et al.*, 1988). However, in a parallel *in situ* study, Creanor and co-workers (1987) could not demonstrate such a fluoride dose-response using the same dentifrices. This result was unexpected and is in disagreement with several other *in situ* studies (Goorhuis & Purdell-Lewis, 1986; de Kloet *et al.*, 1986; Schafer, 1989), where a dose-response relationship was shown using fluoride gels and sodium fluoride or sodium monofluorophosphate dentifrices respectively.

Several improvements were made to the *in situ* model described by Creanor *et al.* (1987). The first improvement involved replacing gelatin-prepared lesions by solution-prepared lesions. In the comparative *in vitro* study described in Chapter 5, solution-prepared lesions (using a well-defined chemical system) were found to be more responsive towards de-/ remineralisation processes. Further *in vitro* work on solution- and gelatin-prepared lesions (Chapter 6), again showed solution-prepared lesions to be more responsive, since low fluoride levels enhanced remineralisation of solution-prepared lesions but only decreased the demineralisation of gelatin-prepared lesions. Such results suggested that solution-prepared lesions should also be more suitable for *in situ* studies especially when different fluoride vehicles or concentrations were being tested. Another advantage with the solution-prepared lesions was that they had a smaller range of baseline Δz values. Hence, as an *in situ* remineralisation study (Strang *et al.*, 1987) showed that

lesion size had an effect on remineralisation, it was favourable to start with lesions of similar Δz mineral content. A further change in the experimental design was that after microradiography, sections were always re-mounted on the same side of an appliance. By so doing, it was possible to determine if any differences occurred in lesion mineral content between appliance sides in the same individual, as has been observed by some workers (Mellberg, Castrovince & Rotsides, 1986; Mellberg *et al.*, 1988). Finally, in the previous *in situ* study (Creanor *et al.*, 1987) the volunteers numbered only three and were considered to be "dentally motivated". In the current study the number of participants was increased to seven and were described, by the examining clinician, as being more representative of the general population.

The aim of the experiment, reported in this chapter was to use the improved *in situ* model to determine whether the *in situ* remineralisation rate of solution-prepared lesions was related to the fluoride concentration of the SMFP dentifrices used by Creanor *et al.* (1987). In addition, several parameters associated with *in situ* remineralisation were measured (i) in order to dentally classify the volunteers, and (ii) as part of a long-term project to correlate various factors such as salivary and plaque fluoride levels, with remineralisation rate.

8.2 Materials and methods

8.2.1 Specimen preparation

Artificial carious lesions were prepared on fifty human premolar teeth using the chemical system described in Section 2.2.3. Sections were cut, ground and varnished as detailed in Chapter 2. The varnished sections with the exposed outer enamel containing the artificial lesions were then mounted in the troughs of *in situ* appliances.

8.2.2 Appliance design

Lower removable *in situ* appliances (Fig. 8.1) were designed to fit either dentate or partially dentate arches. The appliances were similar to those used by Creanor and co-workers (1987). However, instead of an all-acrylic design, those used in this study had a cobalt-chromium framework. For each volunteer, upper and lower alginate impressions (Xantalgin, Bayer, Newbury, England) were taken. From these impressions, models were poured in dental stone and undercut areas present on the lower casts blocked out with plaster. A cobalt-chromium framework was constructed using standard techniques. Acrylic (Trevalon C, De Trey Divison, Dentsply Ltd, Surrey, England) lingual flanges were added to the appliance framework, these carrying the recessed troughs on which the experimental enamel samples were mounted. An inlet and an outlet was cut in the upper and lower edges of the trough to permit the free flow of saliva through this area. Sections were secured in the trough by means

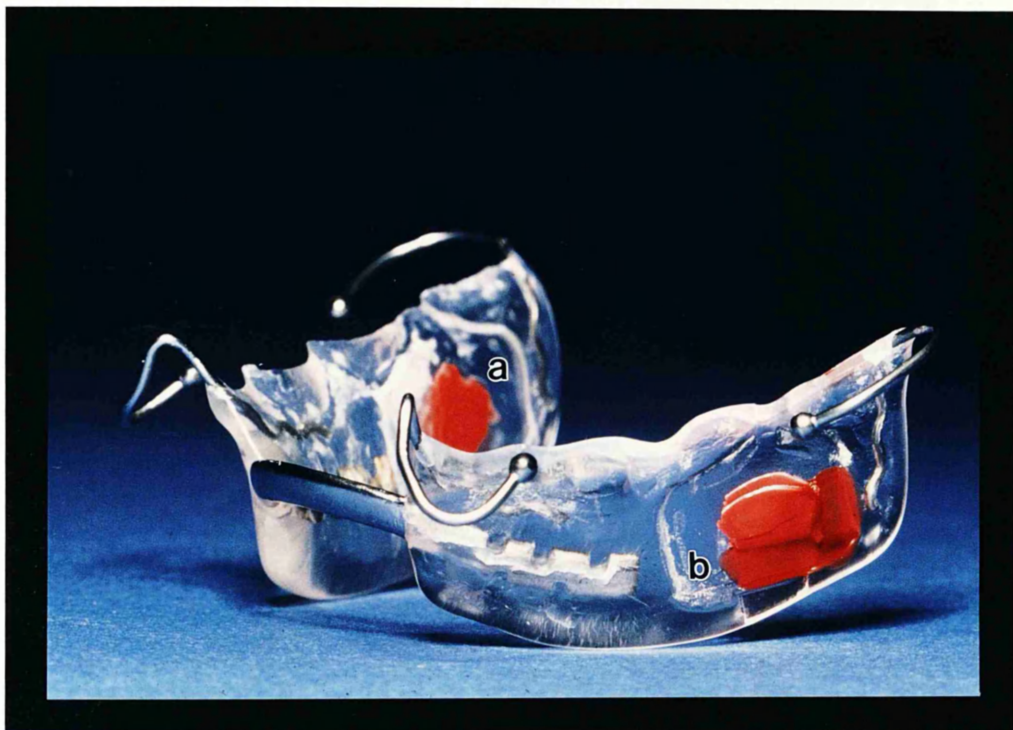


Figure 8.1 A typical in situ appliance as worn by volunteers. The varnished enamel sections were placed in troughs (a) and (b)

of nail varnish, so that when the appliance was positioned in the mouth the sections lay adjacent to the mandibular lingual mucosa.

8.2.3 Volunteers

Appliances were made for sixteen healthy, dentate volunteers working in Glasgow Dental School. A trial run was carried out to assess volunteer co-operation as compliance is crucial in such a study. Those subjects who were uncertain about following the experimental protocol were then excluded from the study. As a result, only seven (two females and five males) of the original sixteen volunteers completed the project.

8.2.4 Test dentifrices

The sodium monofluorophosphate (SMFP) dentifrices were supplied by Unilever Dental Research, Port Sunlight, England. They contained alumina trihydrate as abrasive and were of identical formulation except for the fluoride concentration. The non-F paste contained no added fluoride and was used as a control whereas the two fluoridated dentifrices contained 0.76 % SMFP or 1.9 % SMFP, equivalent to 1000 ppm and 2500 ppm fluoride respectively. The free fluoride content in the fluoridated pastes was approximately 3 % of the nominal fluoride concentration (Schafer, 1989). All pastes were in identical packages apart from a number code.

8.2.5 Experimental design

The experiment was designed to be single-blind and of the cross-over type. With this design, the subjects act as their own control and, unlike a two-group study, fewer participants are required as this method has the advantage of removing variability between subjects (Bland, 1988). The volunteers were randomly allocated to two groups. Group 1 used the 1000 ppm F MFP paste during the first four week test period and the 2500 ppm F paste during the second four week period. Group 2 used the two dentifrices in reverse order. At the end of this cross-over study, only six of the seven volunteers agreed to use a non-F paste in a third run.

Subjects were supplied with a toothbrush (OralB Plus P - 35, Laboratories International, UK.) and the coded dentifrices. They were given written instructions (Appendix II) both on oral hygiene procedures and on how to clean the appliance without disturbing the plaque accumulating in the trough area.

Two or three sections were mounted on each side of the appliance giving a maximium of six sections with 20 lesions on each appliance. The mineral content of the lesions was measured prior to the experiment by means of microradiography and computerised microdensitometry (Chapter 2) and at two-weekly intervals thereafter. At the end of every two weeks the appliances were collected from the volunteers, and the varnished sections (removed from

the trough by means of amyl acetate) were re-microradiographed, then varnished again. These sections were re-mounted on the same side of the appliance and returned to volunteers the same day. For each experimental run, the total number of lesions initially allocated for volunteers was 112.

In order to minimize any long-term carry-over effect, a run-in period of two weeks, using the coded dentifrice, was included before the start of every experimental period.

8.2.6 Statistical analyses

Statistical tests included three way analysis of variance in which remineralisation rates (i) for the three pastes, (ii) between volunteers, and (iii) between the right and left side of the appliance were compared. Two factor interactions between these effects were also investigated. Multiple range analysis tests were performed to determine statistical significances between the pastes, the volunteers, and the right and left side of each appliance. Paired t-tests were used to determine whether salivary and plaque fluoride concentrations for the two fluoridated pastes were significantly different, and to determine any significant differences between plaque weight and plaque fluoride from the two sides of each appliance.

8.2.7 Additional measurements

In order to determine if any of the volunteers had abnormal indices, and since other variables in the complex oral environment are thought to play an important role in *in vivo* remineralisation (vide infra), the following additional information was obtained during the course of the study.

(a) Oral hygiene index scores (OHI), calculus scores and scores on decayed, missing, filled, surfaces and teeth ie. DMFS and DMFT

The modified Greene and Vermillion method (Greene & Vermillion, 1964) was used to measure OHI. The calculus score was assessed on one occasion using the technique described by Volpe, Manhold and Hazen, (1965). DMFS and DMFT values were obtained using standard clinical-only techniques ie. with a plane NO.5 mirror and ball-ended CPITN probe.

(b) Diet, protocol compliance questionaires and paste usage

Volunteers were asked to keep a record of all food intakes during a three day period, including at least one weekend day. This method has been found sufficient for qualitative analysis (Nikiforuk, 1985). At least two diet record sheets (Appendix III) were given to each volunteer for every experimental run.

At the end of the study, a questionaire on protocol

compliance (Appendix IV) was given to each volunteer. It consisted of simple questions to ensure that volunteers were happy with the intra-oral device, but also sought to obtain some information regarding their oral habits prior to this study.

On three separate occasions, each volunteer was given a pre-weighed tube of toothpaste and asked to squeeze out the amount of dentifrice they would normally use. Each tube was re-weighed and the weight difference taken as the amount of paste used per brushing.

(c) Salivary buffer pH

Paraffin-stimulated saliva was obtained two hours after food intake as described below. Using the method described by Krasse (1985), three mL of five mM hydrochloric acid was added to one mL of sample and shaken to remove carbon dioxide. The sample was allowed to stand for ten minutes and the buffer pH of the samples measured on the Ionalyser (Orion 901) using a pH and reference electrode. This procedure was repeated on three occasions.

(d) Salivary flow rate

Traditionally, several methods have been used to collect and measure whole mouth saliva (Kerr, 1961; White, 1977). In this study, a spitting procedure was used to collect unstimulated saliva. With this technique, volunteers were seated comfortably and asked to swallow prior to beginning

a collection. They were then asked to spit into sterile pre-weighed universal bottles, every half minute for five minutes, the saliva which collected behind closed lips. For the stimulated collection, salivary flow was encouraged by the chewing of paraffin wax pellets (Orion, Diagnostica), and saliva collected every half minute, again for five minutes. In both cases, salivary flow rates were determined by weight and not by volume as such measurements are thought to be less reliable (Navazesh & Christensen, 1982). For each volunteer, the stimulated and unstimulated salivas were collected on three separate occasions and always at the same time of day (around 15.00 hours).

(e) Salivary fluoride levels

The samples collected for the measurement of stimulated and unstimulated salivary flow rates were stored frozen for further analyses. Salivary fluoride levels were measured by the indirect method as described in Section 2.4.2. Since the reported fluoride concentration in saliva ranges from 0.009 - 0.05 ppm (Yao & Gron, 1970; Bruun & Givskov, 1979; Bruun *et al.*, 1982; Bruun & Thylstrup, 1984), concentrated TISAB 111 was used to improve the accuracy of fluoride detection in the samples.

(f) Salivary Calcium

Volumetric analysis was used to determine total salivary calcium concentration. The technique was based on the method used to determine the hardness of water, the

"Trilon B" method (Schwarzenbach, 1946). Two mL of five mM EDTA (ethylene diamine tetraacetic acid) and two mL of ammonium buffer were added to five mL of stimulated saliva. A few milligrams of solochrome black / sodium chloride (prepared by mixing 0.1 g solochrome black and 20 g sodium chloride) was added as an indicator and this titrated with five mM magnesium sulphate heptahydrate. The end-point was indicated by a wine-red colour. The concentration of calcium in millimoles per litre was then calculated after calibration of magnesium sulphate with five mL of five mM EDTA.

(g) Plaque fluoride

The method of Duckworth, Morgan & Murray, (1987) was employed to estimate plaque fluoride. At the end of the second or fourth week of every experimental run, plaque was collected in pre-weighed micro-tubes from the right and left trough areas of each appliance and stored frozen at -4°C. At least two mg of plaque was collected with a sterile spatula. Using a 100 µL micro-syringe (Scientific Engineering PTY LTD, Australia), 30 µL of 60 % perchloric acid was added to each plaque sample. The microtubes were capped tightly and left at room temperature for about 20 hours. Each sample was then neutralised with 100 uL solution containing 10 % sodium hydroxide and two % CDTA (1,2 - diaminocyclohexane N,N,N′, - tetraacetic acid). and buffered to pH 5 with 100 µL TISAB 111 and one mL double-distilled deionised water added. Samples were placed in micro-wells and fluoride determined as

described in Chapter 2 (with standards being prepared the same way as the plaque samples). The amount of fluoride in the plaque was expressed as nanogram of plaque fluoride per milligram of plaque wet weight.

8.3 Results - remineralisation

8.3.1 Introduction

In this section, the effect of fluoride concentration (i.e. paste), appliance side and volunteer variation on mineralisation rates will be presented separately (Sections 8.3.2 - 8.3.4). This will be followed by the statistical evaluation which will include multiple range tests. In Section 8.3.6, the results of the two-factor interactions between paste and volunteer, paste and appliance side and between volunteer and appliance side will be described, in relation to the 158 lesions which were suitable for analysis, at the end of the experiment. The remaining lesions had to be excluded because of damage, cavitation or inappropriate size. The mean baseline Δz, surface zone and lesion body values are shown in Table 8.1.

8.3.2 Effect of paste on lesion remineralisation

A summary of the effects of the three pastes on lesion remineralisation rates for Δz, surface zone (SZ), and lesion body (LB) parameters are demonstrated in Figures 8.2 - 8.4. The mean ($\pm$ 95 % confidence limits) for the Δz remineralisation rates (for all pastes and all

Table 8.1 Mean (SE) baseline values for Δz, surface zone (SZ) and lesion body (LB)

Volunteer	Δz (% vol. min. xμm)	SE	SZ (% vol. min.)	SE	LB (% vol. min.)	SE	n
(a) non-F paste							
B	2800	154	60.7	1.3	40.0	2.7	9
D	2984	92	59.8	1.1	46.7	1.2	8
L	2980	291	58.7	2.8	44.7	2.7	6
K	2681	152	61.8	1.3	49.8	1.4	11
P	2693	208	62.3	1.3	49.2	2.1	9
F	3064	208	56.3	2.3	39.6	2.3	8
(b) 1000 ppm F as MFP paste							
B	2962	252	58.2	2.0	45	2.6	10
D	2815	329	61.2	2.0	47	3.9	7
L	2668	276	63.6	2.4	43	2.4	6
K	2894	221	55.0	1.7	40	3.0	8
P	2668	275	57.3	3.7	43	4.4	6
J	2873	249	59.0	3.1	39	3.5	4
F	3541	160	59.7	4.4	38	3.9	9
(b) 2500 ppm F as MFP paste							
B	3232	221	61.3	1.2	41.9	2.9	9
D	3131	132	57.6	1.6	42.4	2.0	11
L	3545	168	55.2	2.6	36.8	1.5	6
K	3330	351	59.2	2.1	45.6	1.4	5
P	3363	215	52.3	1.6	40.7	2.4	11
J	3272	204	56.1	1.5	44.5	1.9	8
F	3067	147	57.4	1.7	43.6	2.1	7

SE = Standard Error of the mean

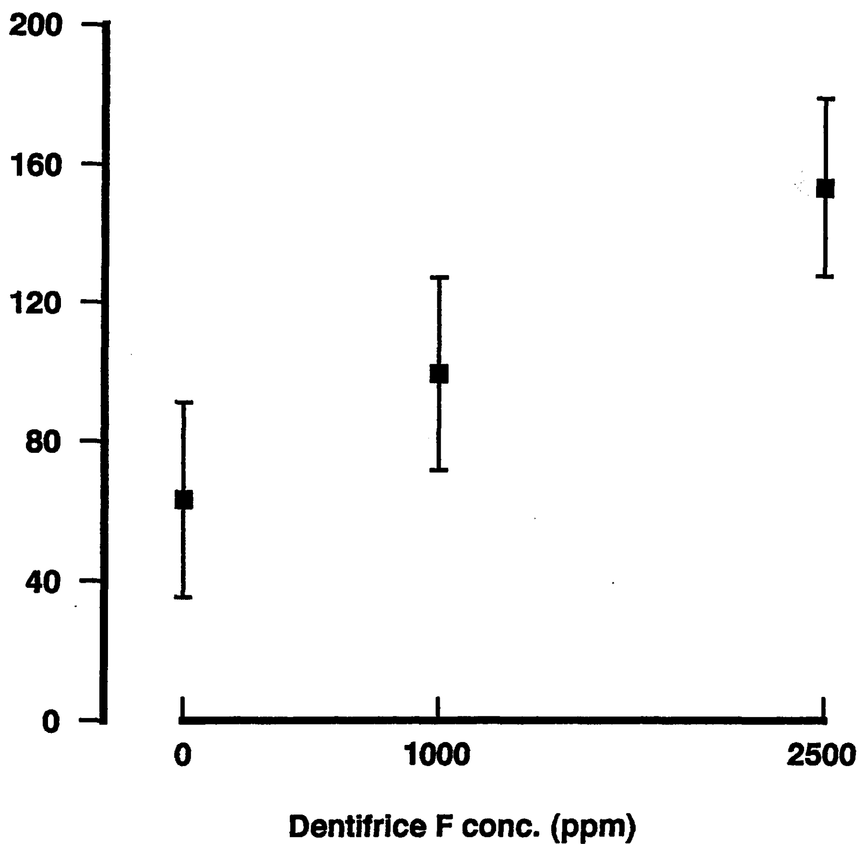


Figure 8.2 95 % confidence intervals for mean Δz remineralisation rates for the three pastes, for all volunteers and both sides of the appliance

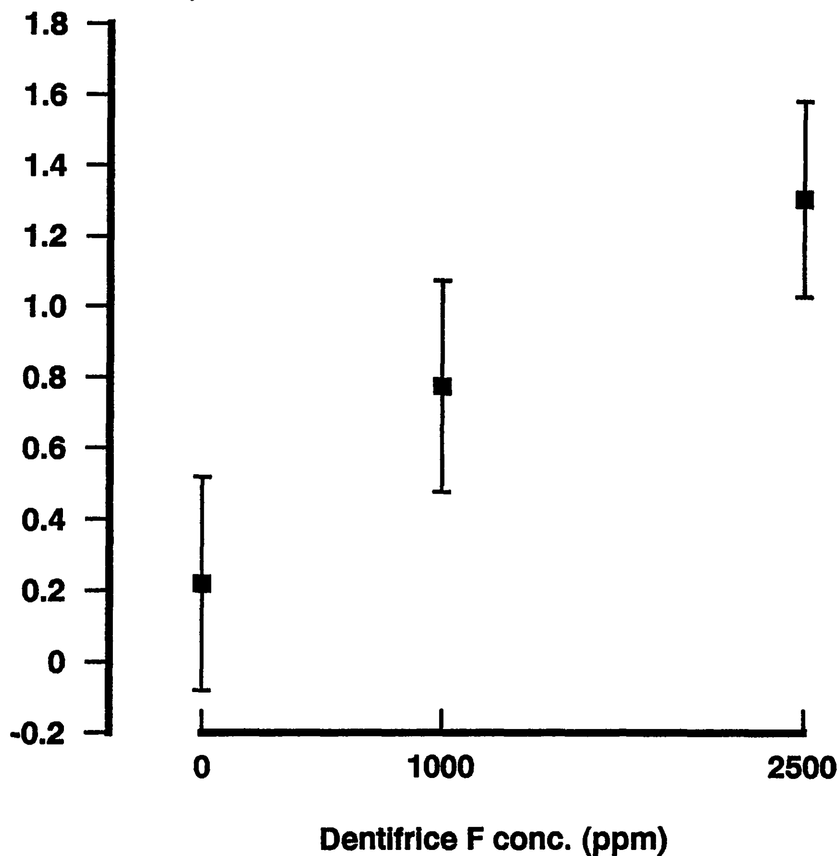


Figure 8.3 95 % confidence intervals for mean surface zone (SZ) mineralisation rates for the three pastes, for all volunteers and both sides of the appliance
+ve values = remineralisation
-ve values = demineralisation

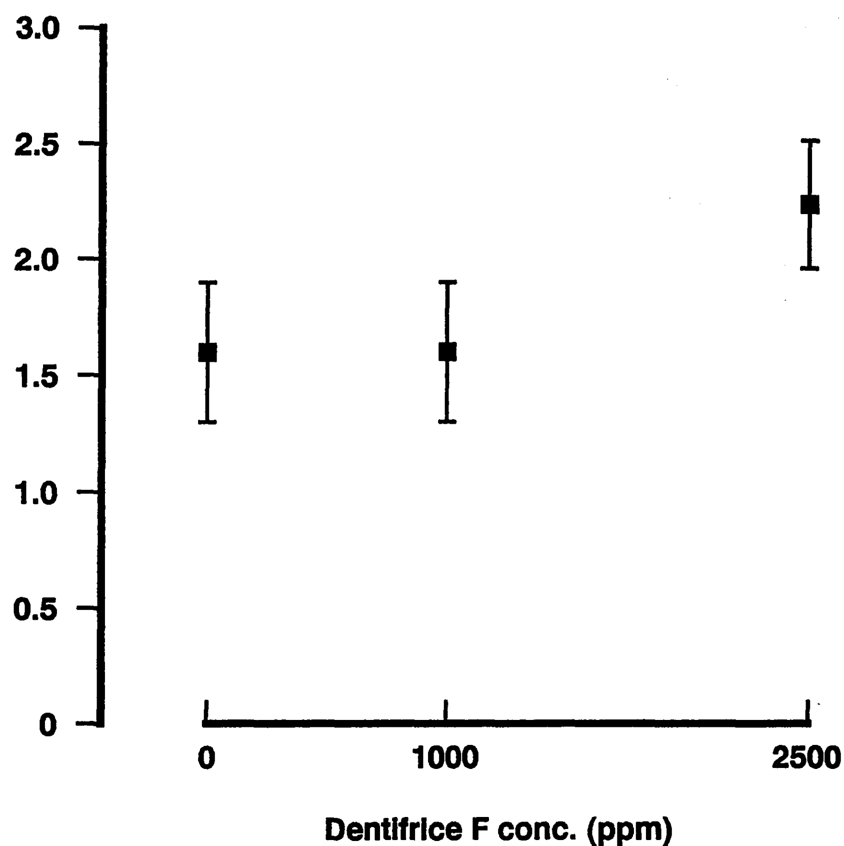


Figure 8.4 95 % confidence intervals for mean lesions body (LB) remineralisation rates for the three pastes, for all volunteers and both sides of the appliance

volunteers increased from 63 (± 28) to 100 (± 28) to 154 (± 26) % vol. min. x μm / wk for the non-F, 1000 and 2500 ppm fluoride respectively. The coresponding values for the surface zone parameter were 0.22 (± 0.30), 0.78 (± 0.30), and 1.32 (± 0.28) % vol. min. / wk and values for the lesion body parameter were 1.61 (± 0.30), 1.62 (± 0.30) and 2.25(± 0.28) % vol. min / wk. The statistical evaluation of these results is discussed in Section 8.3.5.

8.3.3 Effect of appliance side on lesion remineralisation

Figures 8.5 - 8.7 show the Δz, surface zone and lesion body mineralisation rate for lesions from the right and left sides of the appliance. The variations between the right and left side for each volunteer and for each paste are shown in Tables 8.2 - 8.4 for all three parameters. For the Δz parameter, the mean value (± 95 % confidence limits) for the right side was 132.4 (± 17.4) and for the left side it was 78 (± 19.2) % vol. min. x μm /wk. The corresponding surface zone remineralisation rate values were 0.56 (± 0.24) and 0.99 (± 0.22) % vol. min. / wk and the lesion body remineralisation rate values were 2.12 (± 0.19) and 1.49 (± 0.21) % vol. min / wk.

8.3.4 Effect of volunteer

The variations in mineralisation rate values among volunteers are shown in Tables 8.5 - 8.7 for the three parameters. The variations among volunteers are also demonstrated in Figures 8.8 - 8.10. The mean (± 95 % confidence limits) Δz remineralisation rates varied

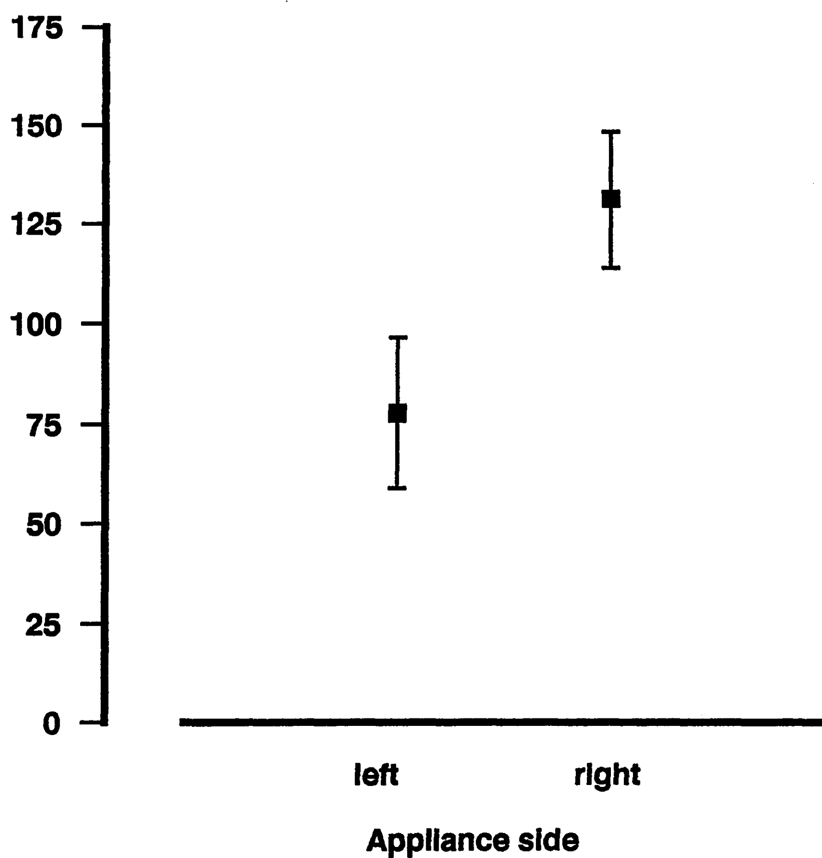


Figure 8.5 95 % confidence intervals for mean Δz remineralisation rates by appliance sides for all volunteers and all pastes

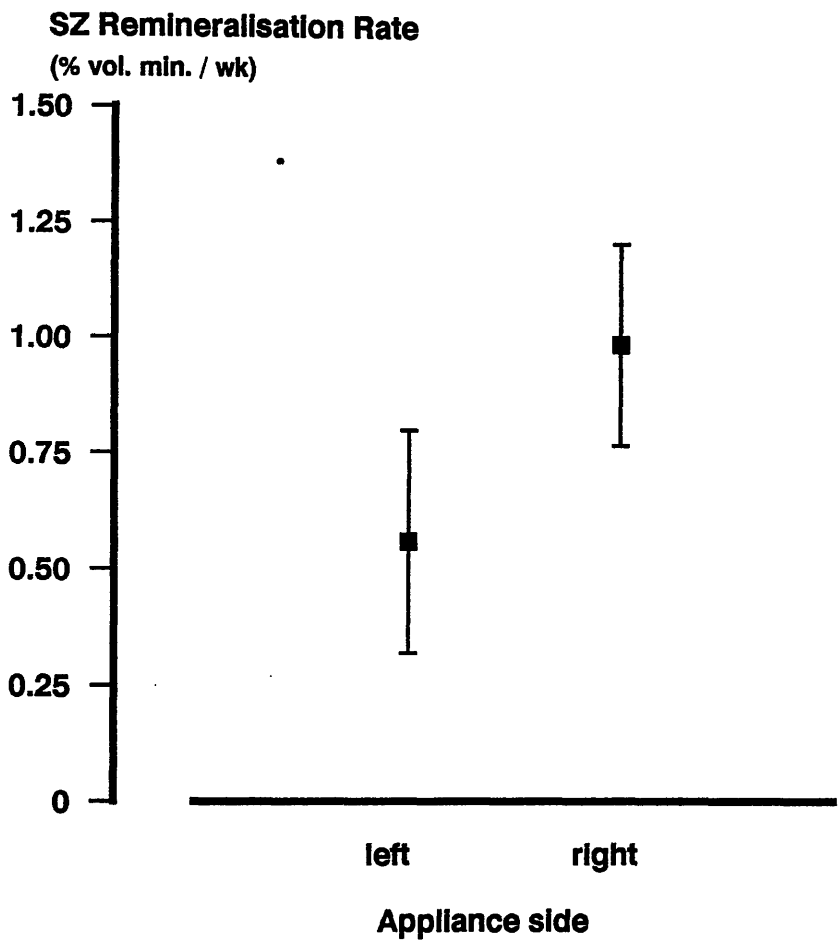


Figure 8.6 95 % confidence intervals for mean surface zone (SZ) remineralisation rates by appliance sides for all volunteers and all pastes

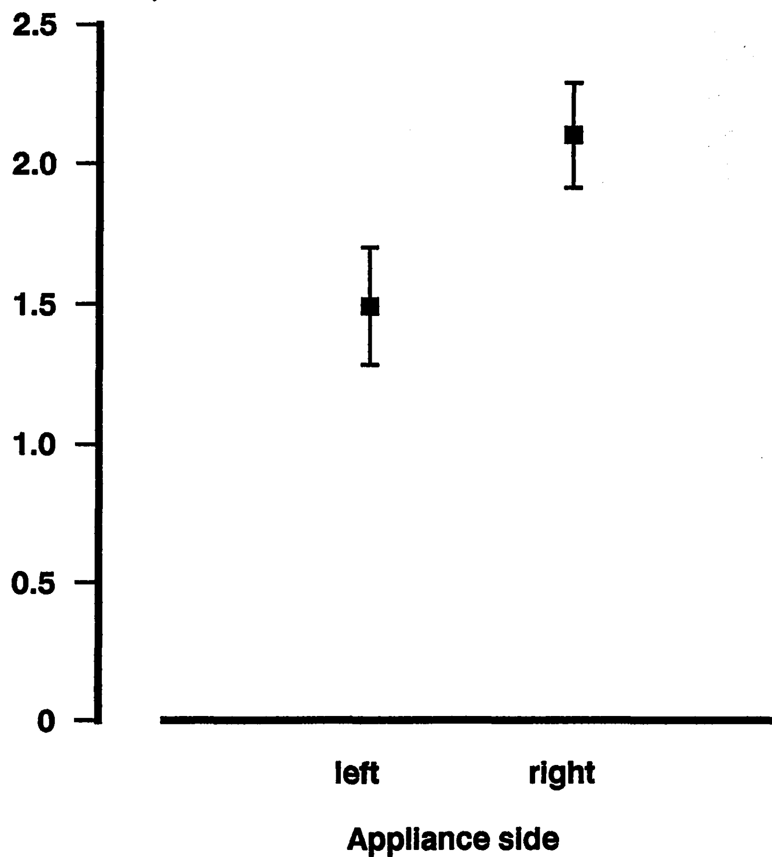


Figure 8.7 95 % confidence intervals for mean lesion body (LB) remineralisation rates by appliance sides for all volunteers and all pastes

Table 8.2 Mean (SE) Δz mineralisation rates for all volunteers when lesions are grouped according to side of the appliance

Volunteer	non-F		1000 ppm F		2500 ppm F	
	Right	Left	Right	Left	Right	Left
B	214	150	192	121	228	147
(SE)	(51)	(19)	(34)	(31)	(39)	(21)
D	196	-6	179	105	165	122
(SE)	(29)	(36)	(33)	(30)	(14)	(17)
L	-68	85	67	81	276	212
(SE)	-	(64)	(24)	-	(14)	-
K	33	-322	100	47	162	46
(SE)	(17)	(81)	(18)	(19)	(43)	(0)
P	110	-	128	55	157	192
(SE)	(23)		(101)	(59)	(44)	(53)
J	-	-	44	54	25	45
(SE)	-	-	(29)	-	(30)	(11)
F	85	85	106	100	175	155
(SE)	(19)	(22)	(19)	(39)	(32)	(12)

SE = Standard Error of the mean

Table 8.3 Mean (SE) surface zone (SZ) mineralisation rates for all volunteers when lesions are grouped according to side of the appliance

Volunteer	non-F		1000 ppm F		2500 ppm F	
	Right	Left	Right	Left	Right	Left
B	0.65	1.36	1.97	1.19	2.20	1.36
(SE)	(0.42)	(0.62)	(0.57)	(0.31)	(0.20)	(0.16)
D	1.20	-0.95	1.80	1.13	1.22	1.08
(SE)	(0.40)	(0.75)	-	(0.23)	(0.19)	(0.39)
L	-0.76	0.53	0.35	-1.03	2.33	1.95
(SE)	-	(0.78)	(0.65)	-	(0.50)	-
K	0.32	-1.98	1.05	0.40	0.95	0.71
(SE)	(0.19)	(0.79)	(0.68)	(0.42)	(0.48)	-
P	0.97	-	0.88	0.93	1.49	1.77
(SE)	(0.35)	-	-	(0.38)	(0.39)	(0.27)
J	-	-	-1.78	0.61	-0.01	0.09
(SE)	-	-	(1.10)	(0.11)	(0.50)	(0.14)
F	0.27	-1.48	1.60	0.41	1.58	1.36
(SE)	(0.25)	(0.45)	(0.27)	(0.63)	(0.42)	(0.19)

SE = Standard Error of the mean

Table 8.4 Mean (SE) lesion body (LB) mineralisation rates for all volunteers when lesions are grouped according to side of the appliance

Volunteer	non-F Right	non-F Left	1000 ppm F Right	1000 ppm F Left	2500 ppm F Right	2500 ppm F Left
B	4.15	3.56	3.64	2.12	3.52	2.14
(SE)	(0.61)	(0.38)	(0.36)	(0.20)	(0.27)	(0.37)
D	3.02	0.15	2.04	1.36	2.35	2.20
(SE)	(0.52)	(0.25)	-	(0.22)	(0.49)	(0.19)
L	-0.08	1.05	1.49	1.63	3.92	4.15
(SE)	-	(0.63)	(0.60)	-	(0.27)	-
K	0.48	-1.30	1.52	0.97	1.80	0.74
(SE)	(0.23)	(0.33)	(0.24)	(0.38)	(0.21)	-
P	1.91	-	2.31	0.40	2.59	2.59
(SE)	(0.27)	-	-	(0.56)	(0.54)	(0.60)
J	-	-	-0.12	1.57	0.17	0.31
(SE)	-	-	-	-	(0.25)	(0.17)
F	1.65	2.21	1.42	1.43	2.63	1.72
(SE)	(0.31)	(0.30)	(0.49)	(0.39)	(0.45)	(0.32)

SE = Standard Error of the mean

Table 8.5 In situ Δz, surface zone (SZ), lesion body (LB) de / remineralisation rates for the non-F paste

	Δz (% vol. min. x µm / wk)	SE	SZ (% vol. min. / wk)	SE	LB (% vol. min. /wk)	SE
	De- / Remineralisation Rates*					
Subject B (n=9)						
	359	33	1.38	2.2	5.35	8.7
	290	67	1.59	1.14	5.68	0.53
	207	36	-0.74	0.90	4.07	0.58
	71	134	0.33	0.36	3.13	1.45
	146	78	0.69	0.85	2.52	0.95
	107	10	-0.04	0.23	4.14	0.23
	193	26	2.12	0.54	3.74	0.64
	172	50	2.65	0.01	3.93	1.41
	129	110	0.69	1.20	2.44	0.77
mean	186	30	0.96	0.30	3.88	0.37
Subject D (n = 8)						
	167	169	1.45	1.13	2.38	1.99
	132	40	1.33	0.47	2.15	0.79
	259	38	1.96	0.28	4.48	0.19
	227	118	0.06	0.66	3.07	1.33
	-102	84	-3.19	1.18	0.49	2.20
	63	53	-0.31	0.01	-0.01	0.09
	-14	46	-0.37	0.45	0.44	0.44
	30	11	0.05	0.23	0.64	0.24
mean	95	44	0.12	0.57	1.58	0.60
Subject L (n = 6)						
	49	16	0.55	0.20	0.54	0.10
	-49	18	-1.16	0.37	-0.28	0.14
	14	76	0.02	1.11	0.25	0.75
	135	132	0.70	1.69	2.36	0.72
	-185	60	-2.07	1.00	-0.70	0.20
	241	58	2.57	0.94	1.87	0.23
mean	34	60	0.10	0.66	0.67	0.49

* +ve values = remineralisation
-ve values = demineralisation
SE = Standard Error

Table 8.5 (continued)

	De- / Remineralisation Rates*					
	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x μm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
Subject K (n=11)						
	47	98	0.91	1.65	0.84	1.21
	22	17	-0.31	0.06	0.72	0.29
	78	37	0.65	0.12	0.93	0.40
	61	31	0.84	0.87	0.57	0.63
	72	168	0.45	1.93	1.06	1.91
	-44	19	-0.07	0.08	-0.31	0.32
	-2	57	-0.23	0.98	-0.48	1.05
	-208	147	-0.55	0.19	-0.76	0.57
	-200	160	-1.27	0.89	-0.71	0.51
	-335	176	-4.21	2.12	-2.02	1.33
	-548	285	-1.89	0.63	-1.69	0.50
mean	-96	61	-0.52	0.45	-0.17	0.32
Subject P (n = 9)						
	162	27	1.41	0.56	2.36	0.45
	117	55	1.31	0.68	2.19	0.54
	163	44	1.30	0.11	2.63	0.45
	195	17	1.30	0.66	3.15	0.31
	46	14	-1.14	0.47	1.36	0.68
	169	112	2.77	0.66	2.36	1.28
	-2	36	0.25	0.09	0.64	0.63
	50	113	0.35	1.37	1.06	0.84
	91	24	1.20	1.20	1.40	0.49
mean	110	22	0.97	0.36	1.91	0.27
Subject F (n = 8)						
	135	80	-0.13	0.38	2.35	1.07
	24	46	-0.43	0.83	1.31	0.64
	109	74	0.62	0.82	2.47	0.85
	82	30	0.97	0.39	1.04	0.36
	72	13	0.31	0.37	1.08	0.16
	45	75	-2.03	1.31	2.31	0.89
	89	37	-1.83	1.16	1.64	0.72
	120	103	-0.59	1.72	2.67	1.14
mean	85	13	-0.39	0.38	1.85	0.24

* +ve values = remineralisation
-ve values = demineralisation
SE = Standard Error

Table 8.6 In situ Δz, surface zone (SZ), lesion body (LB) de / remineralisation rates for the 1000 ppm F as MFP paste

	Δz	SE	SZ	SE	LB	SE
	De- / Remineralisation Rates*					
	(% vol. min. x µm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
Subject B (n=10)						
	232	28	0.82	0.73	3.48	1.08
	157	173	0.57	1.29	2.71	1.85
	307	160	3.54	0.82	4.75	0.74
	142	30	2.79	0.49	3.12	0.86
	120	101	2.11	1.00	4.12	0.65
	186	88	1.82	0.78	2.49	0.79
	185	51	1.59	0.59	1.87	0.47
	35	76	1.60	0.10	1.80	0.17
	62	74	0.15	1.13	1.73	0.74
	137	86	0.79	0.48	2.72	1.11
mean	156	25	1.58	0.33	2.88	0.32
Subject D (n = 7)						
	211	67	1.75	0.71	2.35	0.59
	146	35	1.85	0.38	1.73	0.66
	35	14	0.50	0.15	1.16	0.62
	78	81	0.66	1.08	1.29	0.84
	216	2	1.61	0.33	1.66	0.55
	89	10	1.59	0.09	0.70	0.00
	106	54	1.28	0.43	1.98	1.18
mean	126	26	1.32	0.20	1.55	0.20
Subject L (n = 6)						
	7	11	-1.09	1.12	0.17	0.41
	47	6	-0.39	0.34	1.96	0.37
	114	59	1.43	1.87	2.90	0.27
	101	19	1.48	0.36	0.92	0.92
	50	99	-1.56	2.30	0.80	1.14
	111	64	-0.49	0.56	2.45	0.28
mean	72	18	-0.01	0.50	1.53	0.40

* +ve values = remineralisation
-ve values = demineralisation
SE = Standard Error

Table 8.6 (continued)

	De- / Remineralisation Rates*					
	Δz (% vol. min. x µm / wk)	SE	SZ (% vol. min. / wk)	SE	LB (% vol. min. /wk)	SE
Subject K (n = 8)						
	97	40	0.00	2.03	1.46	0.64
	71	16	0.83	0.15	1.97	1.12
	132	19	2.32	0.80	1.18	0.08
	51	108	0.74	1.55	1.11	1.29
	67	56	0.25	0.10	2.25	0.78
	73	115	-0.10	1.39	0.63	1.18
	71	25	1.82	0.82	0.91	1.49
	-28	18	-0.69	0.13	-0.06	0.33
mean	66.8	16	0.65	0.36	1.17	0.26
Subject P (n = 6)						
	27	41	0.88	0.76	0.75	0.59
	229	89	0.87	1.54	3.86	1.60
	22	16	0.50	0.31	-0.07	0.39
	-40	43	0.14	0.69	-0.41	0.53
	11	25	1.25	0.23	0.02	0.09
	228	50	1.82	0.64	2.05	0.53
mean	80	48	0.91	0.55	1.03	0.67
Subject J (n = 4)						
	55	4	-0.49	1.02	0.40	0.45
	-11	35	-3.93	0.71	0.76	0.11
	87	142	-0.93	1.88	0.66	1.56
	54	69	-0.12	1.13	1.57	1.00
mean	46	21	-1.37	0.87	1.85	0.25
Subject F (n = 9)						
	122	20	2.05	0.90	1.20	1.08
	185	33	1.83	0.86	2.02	1.38
	109	19	0.83	0.60	2.94	0.86
	141	4	1.08	1.11	2.18	0.62
	12	41	-0.48	0.88	0.78	0.32
	154	49	2.37	1.39	1.70	0.47
	112	26	1.33	0.52	1.36	0.43
	63	26	-0.81	0.72	0.72	0.30
	36	10	1.38	1.00	-0.12	0.12
mean	104	19	1.06	0.36	1.42	0.30

* +ve values indicate remineralisation
-ve values indicate demineralisation
SE = Standard Error

Table 8.7 In situ Δz, surface zone (SZ), lesion body (LB) de / remineralisation rates for the 2500 ppm F as MFP paste

	Δz	SE	SZ	SE	LB	SE
	(% vol. min. x µm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
	De- / Remineralisation Rates*					
Subject B (n=9)						
	231	73	1.70	0.83	3.31	0.43
	319	13	2.90	0.11	4.37	0.78
	160	80	2.16	0.35	3.74	0.03
	129	54	1.99	0.71	2.71	1.07
	109	164	1.28	1.70	1.51	1.86
	141	30	1.74	0.37	1.67	0.80
	132	56	1.44	0.20	2.25	0.56
	305	6	2.24	0.01	3.49	1.03
	207	33	0.96	0.71	3.13	0.07
mean	193	26	1.82	0.19	2.90	0.32
Subject D (n = 11)						
	129	14	0.97	0.15	1.39	0.03
	168	90	1.79	1.57	3.07	1.02
	196	90	1.16	0.86	3.31	0.86
	167	33	0.87	0.42	1.62	0.28
	56	20	1.53	0.41	2.78	0.61
	119	14	2.29	0.31	2.23	0.64
	131	79	2.34	0.73	2.14	0.72
	164	46	1.20	0.58	2.16	0.37
	85	19	0.12	0.74	1.29	0.24
	114	123	0.25	1.85	2.09	1.26
	186	42	0.09	0.97	2.71	0.63
mean	138	13	1.12	0.25	2.25	0.20
Subject L (n = 6)						
	309	32	3.78	0.02	4.49	0.64
	241	54	2.13	1.11	3.19	0.13
	283	89	1.72	0.51	3.98	1.51
	270	90	1.70	0.74	4.01	1.31
	205	103	2.44	2.29	5.38	0.16
	219	196	1.46	1.73	2.92	2.15
mean	255	16	2.21	0.35	3.99	0.36

* +ve values = remineralisation
-ve values = demineralisation
SE = Standard Error

Table 8.7 (continued)

	Δz	SE	SZ	SE	LB	SE
	De- / Remineralisation Rates*					
	(% vol. min. x μm / wk)		(% vol. min. / wk)		(% vol. min. /wk)	
Subject K (n=5)						
	67	98	-0.55	1.29	1.47	1.12
	283	39	2.15	0.76	2.38	0.02
	244	74	1.44	0.08	2.15	0.74
	125	24	1.39	0.59	1.75	0.21
	46	22	0.71	0.22	0.74	1.15
mean	153	40	1.01	0.39	1.69	0.25
Subject P (n = 11)						
	62	86	0.43	0.79	1.10	0.94
	26	37	0.27	1.01	1.16	0.32
	219	126	2.40	0.14	3.21	0.01
	83	28	0.79	0.52	1.53	0.47
	364	24	3.06	0.35	5.02	1.13
	150	36	1.79	0.16	3.21	0.05
	195	37	1.66	0.71	2.89	0.61
	106	100	2.10	0.75	1.67	1.09
	102	134	1.30	1.83	1.67	1.24
	246	202	1.32	1.50	2.82	2.82
	314	140	2.37	1.19	4.18	1.84
mean	170	33	1.59	0.26	2.56	0.39
Subject J (n = 8)						
	13	45	0.23	1.74	0.85	1.84
	-40	48	-0.95	0.10	-0.04	0.68
	20	51	-0.60	0.63	-0.33	0.17
	106	148	1.28	1.66	0.18	1.11
	42	21	-0.09	0.14	0.33	0.11
	78	110	0.32	1.14	0.71	0.88
	27	114	-0.21	1.56	-0.10	0.98
	34	103	0.33	1.04	0.29	0.79
mean	35		0.04		0.24	
Subject F (n = 7)						
	210	15	2.07	0.12	2.05	0.97
	112	22	0.76	0.05	2.32	0.69
	203	3	1.91	0.62	3.51	0.15
	164	124	1.43	1.46	1.76	1.97
	176	146	1.74	1.13	2.29	1.61
	121	99	1.44	1.30	0.81	1.39
	161	7	0.84	1.20	2.02	1.02
mean	164	14	1.54	0.16	2.10	0.3

* +ve values = remineralisation
-ve values = demineralisation
SE = Standard Error

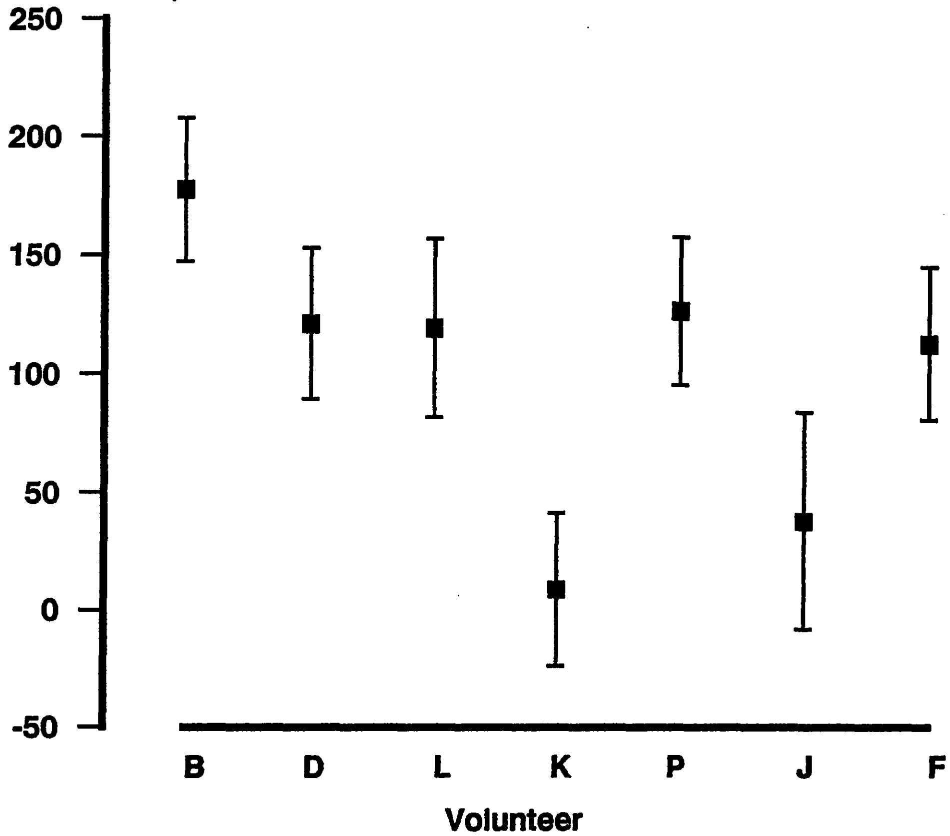


Figure 8.8 95 % confidence intervals for mean Δz mineralisation rates for each volunteer for all pastes and both sides of the appliance
+ve values = remineralisation
-ve values = demineralisation

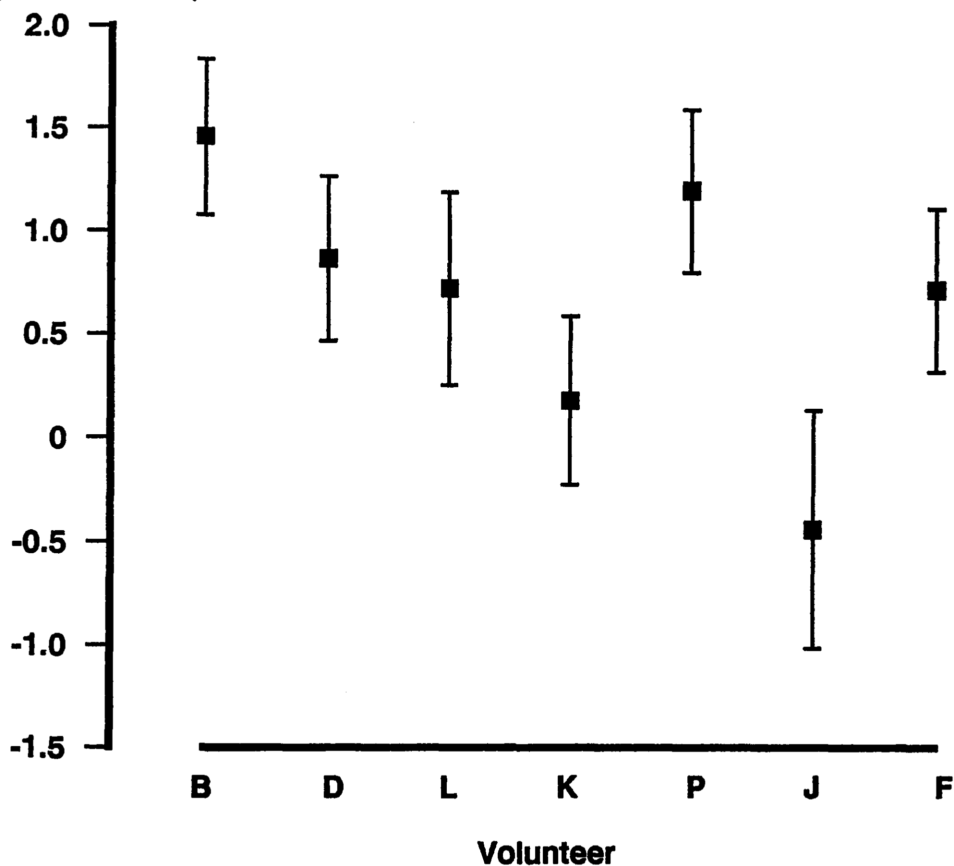


Figure 8.9 95 % confidence intervals for mean surface zone (SZ) mineralisation rates for each volunteer for all pastes and both sides of the appliance
+ve values = remineralisation
-ve values = demineralisation

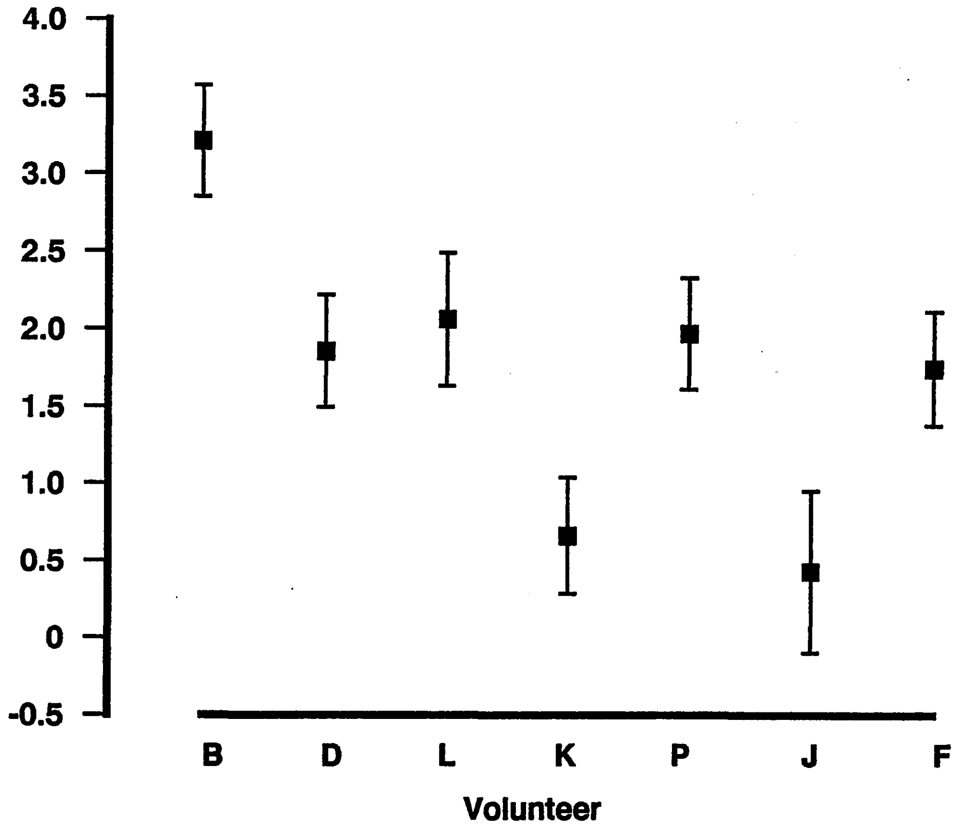


Figure 8.10 95 % confidence intevals for mean lesion body (LB) mineralisation rates for each volunteer for all pastes and both sides of the appliance
+ve values = remineralisation
-ve values = demineralisation

between 9.25 (± 32.9) for volunteer (K) to 178 (± 30.3) % vol. min. x μm / wk for volunteer (B). The values for the surface zone parameter ranged from -0.43 (± 0.58) to 1.46 (± 0.38) % vol. min. / wk and for the lesion body the remineralisation rate ranged from 0.44 (± 0.53) to 3.21 (± 0.36) % vol. min. / wk.

8.3.5 Statistical analyses

Results of the three-way analysis of variance for Δz, surface zone and lesion body mineralisation rate are summarised in Appendix V. For each parameter, the main effects, i.e. pastes, volunteers and appliance side, were statistically significant (Table 8.8), showing that (a) there was a fluoride dose-response (b) the position (i.e. the side of the appliance on which the sections were mounted) affected the mineralisation rates and (3) there were significant differences between volunteers.

Following the significant results obtained from the analysis of variance, multiple range analysis tests were carried out to investigate the significance of individual groups. The mean Δz mineralisation rate for the non-F paste was significantly lower than that for the 2500 ppm fluoride paste. Similarly, the 1000 ppm fluoride paste was significantly lower than the 2500 ppm fluoride paste (Fig. 8.2). However the difference in mineralisation rates for the non-F paste and the 1000 ppm fluoride paste did not quite attain significance. The surface zone and lesion body mineralisation rates showed

Table 8.8 Significance levels in the analysis of variance for Δz, surface zone (SZ) and lesion body (LB) remineralisation rates

	probability, p		
	Δz	SZ	LB
Main effects			
paste	0.00005	0.00005	0.00005
volunteer	0.00005	0.00005	0.00005
side	0.00005	0.0022	0.00005
Two-factor interactions			
paste / volunteer	0.0001	0.0478	0.00005
paste / side	0.1837	0.7512	0.7430
volunteer / side	0.0003	0.1816	0.0113

the same significant differences as the Δz parameter. (Figs. 8.3, 8.4). The range tests carried out to assess significance between right and left appliance side showed a significant difference for the Δz and lesion body parameters (Figs. 8.5, 8.7) but no significant difference for the mean surface zone mineralisation rate (Fig. 8.6).

8.3.6 Two-factor interactions

Two factor interactions (Appendix V) showed significant interactions between paste and volunteers for all three parameters (Table 8.8) indicating that their responses to the increase in dentifrice fluoride concentration were different (Figs. 8.11 - 8.13). However, the differences observed between the right and left sides of the appliance were not dependant on the paste used. The interaction between volunteer and side (Figs. 8.14 - 8.16) was significantly different for the Δz and lesion body remineralisation rates but not for the surface zone values. These significant interactions indicate that the side which gave the better remineralisation rate values differed for the different volunteers.

8.4 Results - additional measurements

8.4.1 Age, OHI, calculus score, DMFS

The volunteers' age, OHI and calculus scores as well as DMFS values for the right and left side of the mouth, are presented in Table 8.9. The ages ranged from 20 - 47

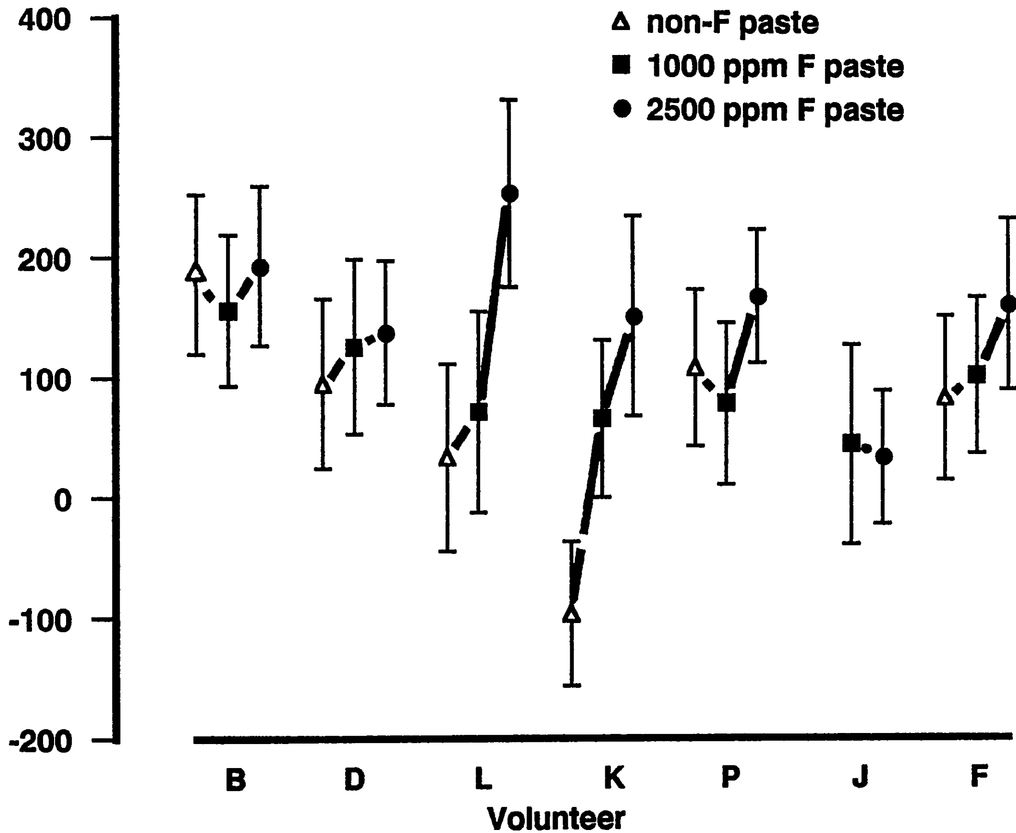


Figure 8.11 95 % confidence intervals for mean Δz mineralisation rate for each volunteer and for each paste, for both sides of the appliance
+ve values = remineralisation
-ve values = demineralisation

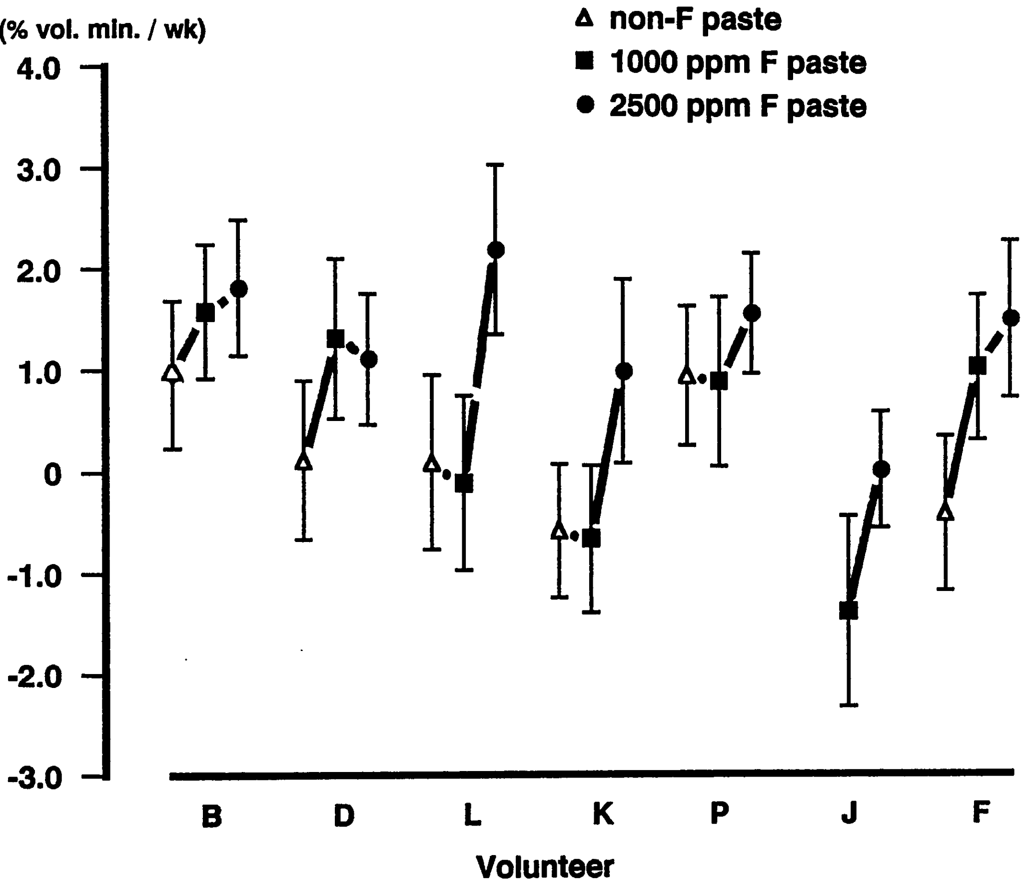


Figure 8.12 95 % confidence intervals for mean surface zone (SZ) mineralisation rate for each volunteer and for each paste, for both sides of the appliance
+ve values = remineralisation
-ve values = demineralisation

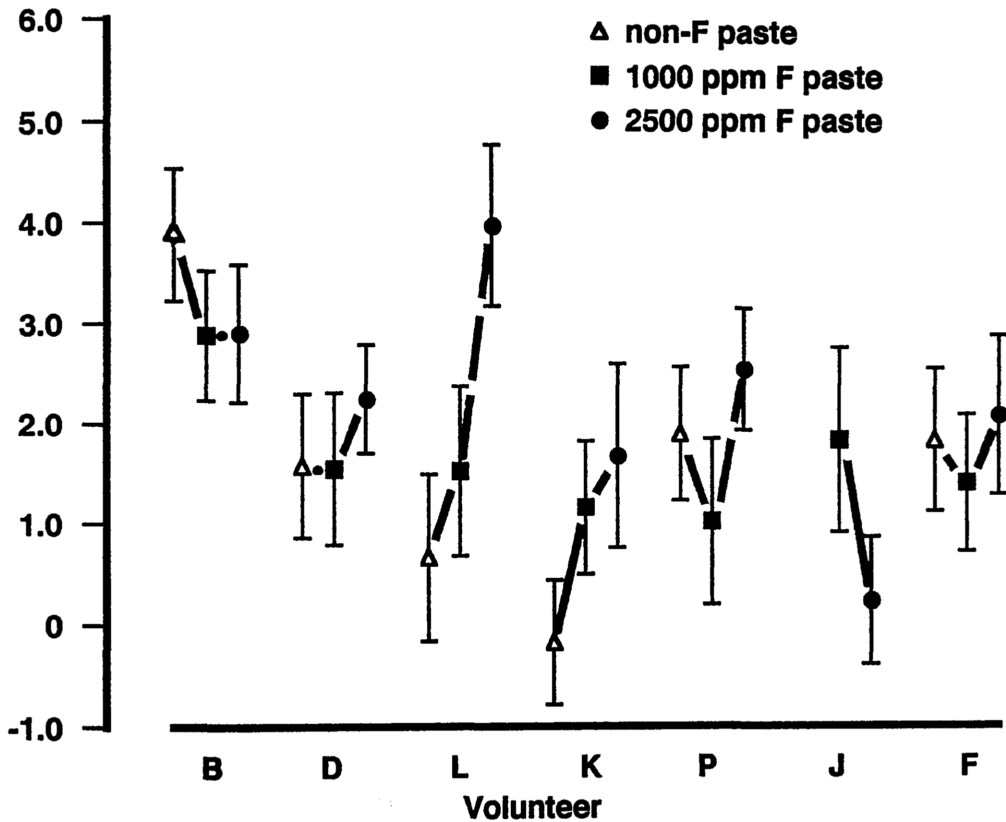


Figure 8.13 95 % confidence intervals for mean lesion body (LB) mineralisation rate for each volunteer and for each paste, for both sides of the appliance
+ve values = remineralisation
-ve values = demineralisation

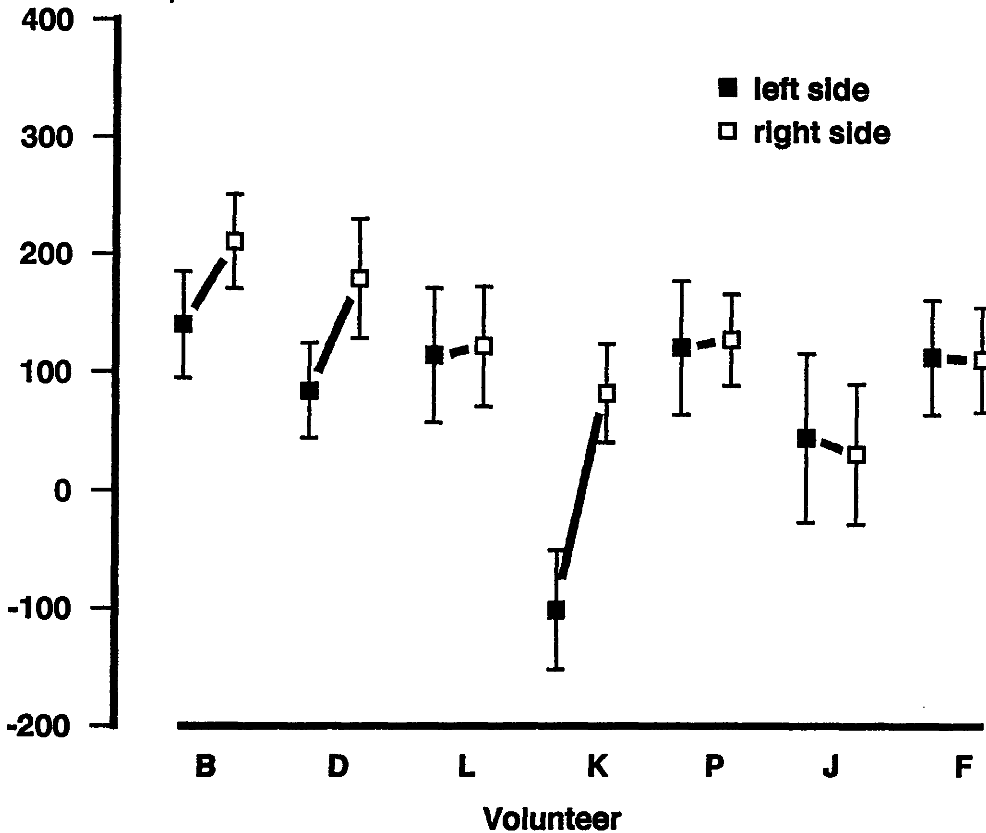


Figure 8.14 95 % confidence intervals for mean Δz mineralisation rates for each side of the appliance and for each volunteer, for all pastes
+ve values = remineralisation
-ve values = demineralisation

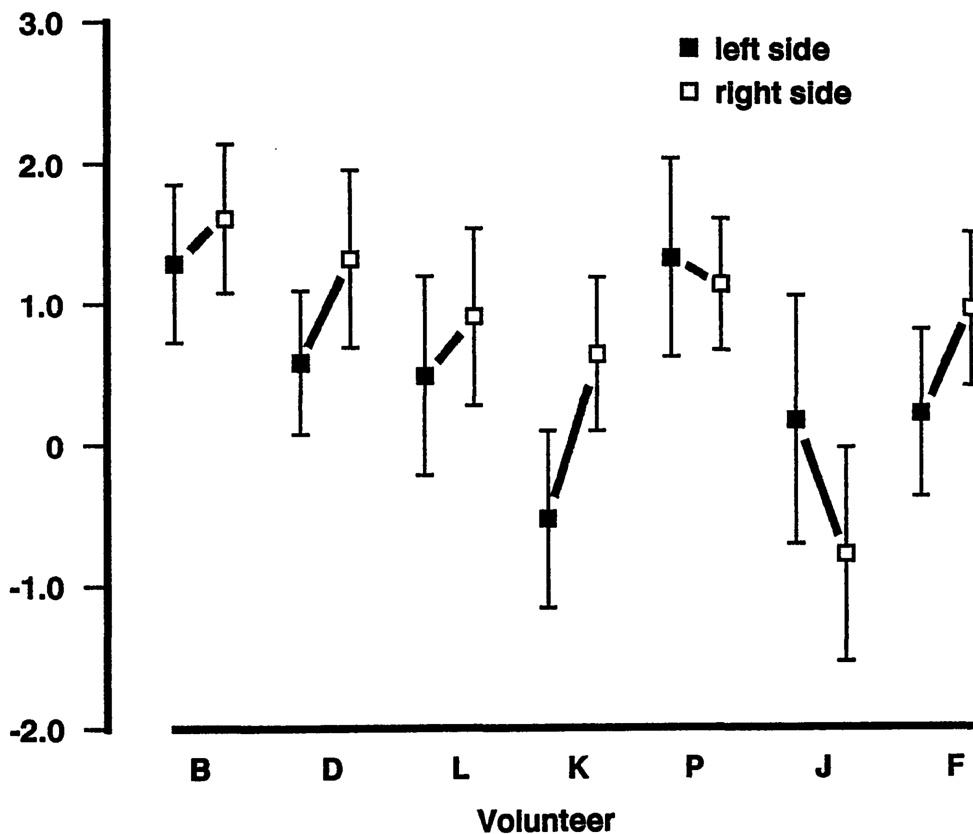


Figure 8.15 95 % confidence intervals for mean surface zone (SZ) mineralisation rates for each side of the appliance and for each volunteer, for all pastes
+ve values = remineralisation
-ve values = demineralisation

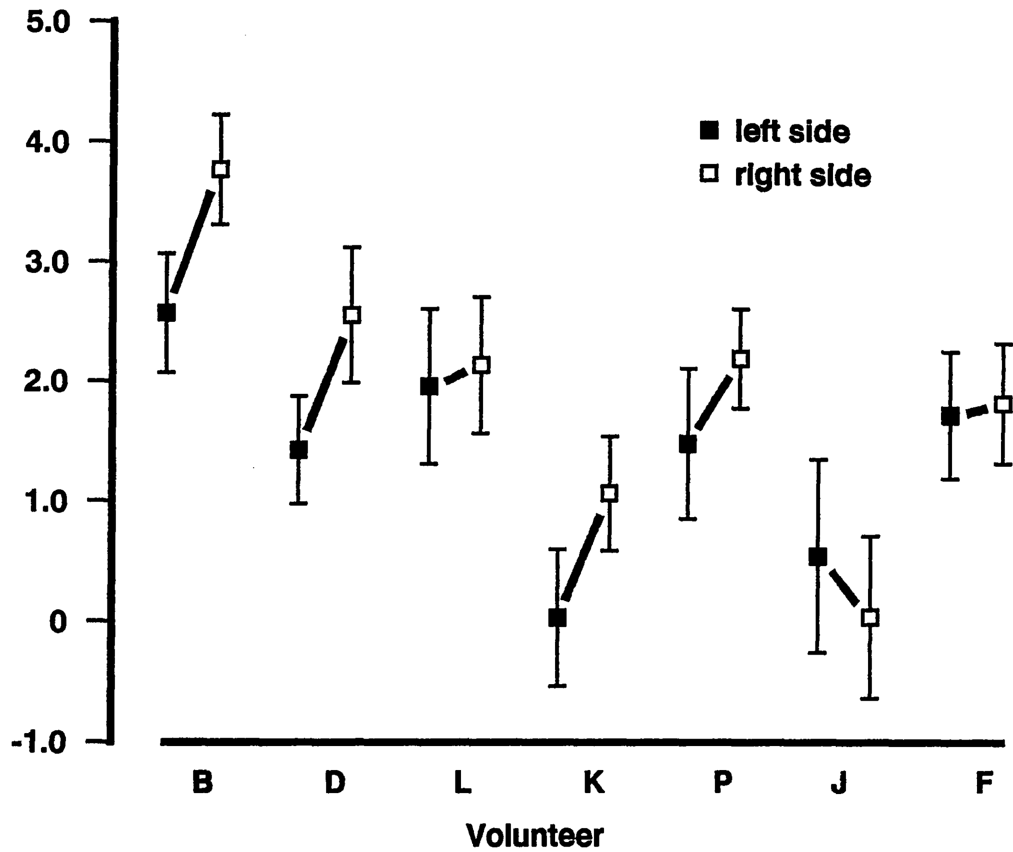


Figure 8.16 95 % confidence intervals for mean lesion body (LB) mineralisation rates for each side of the appliance and for each volunteer, for all pastes
+ve values = remineralisation
-ve values = demineralisation

Table 8.9 Age, DMFS & Calculus Scores, and Oral Hygiene Index (OHI) values for the seven volunteers

Volunteer	Age	DMFS		Calculus score	OHI
		RHS	LHS		
B	41	25	24	12.5	0
D	36	12	9	1.0	1
L	20	13	17	15.0	3
K	35	12	22	0	0
P	47	22	23	2.0	2
J	26	14	11	0	0
F	35	14	13	0	1

DMFS - Decayed Missing Filled Surfaces
OHI - Oral Hygiene Index
RHS - Right Hand Side
LHS - Left Hand Side

years (mean age 34 yrs). Of the volunteers, five were considered to be reasonably dentally motivated as reflected by their low oral hygiene index scores (Table 8.9). Unfortunately, the calculus assessment was only performed on one occasion and, therefore, little information could be obtained about the volunteers' true calculus formation rates. However, values shown in Table 8.9 might suggest that two of the volunteers (B) and (L) could have been heavy calculus depositors. The DMFS values of all but one volunteer (K) were symmetrically distributed. Volunteer (K), with a DMFS on the right side of 12 and a DMFS on the left side of 22, had had previous orthodontic treatment and, therefore, the examining clinician could not confirm whether this volunteer had a true DMFS asymmetry. The data shown in Table 8.9 was used to obtain information regarding the volunteers' "dental status" and no attempt was made to correlate these values with the observed remineralisation rates.

8.4.2 Salivary measurements and paste usage

Values obtained for the volunteers' salivary buffer pH, stimulated and unstimulated flow rate and calcium concentration are shown in Table 8.10. Also included in this table are data relating to weight of paste used per brushing, which varied from 1.11 g - 2.1 g. Volunteers (K) and (P) showed poor buffer pH values of 3.8 and 4.1 respectively. Volunteer (L) and volunteer (D) gave a very low unstimulated (0.13 g/min) flow rate, although the

Table 8.10 Buffer pH, stimulated (S) and unstimulated (U) salivary flow rate, dentifrice usage and salivary calcium concentration of the seven volunteers (mean (SD) of 3 measurements)

Volunteer	Buffer pH	Salivary Flow Rate (g/min) (S)	(U)	Wt Paste/ brushing (g)	Salivarv Ca-ion conc. (mM)
B	6.3	1.41	0.58	1.9	1.05
(SD)		(0.32)	(0.11)	(0.00)	(0.09)
D	5.1	1.36	0.13	1.6	1.16
(SD)		(0.06)	(0.02)	(0.03)	(0.35)
L	5.5	0.53	0.13	1.11	1.15
(SD)		(0.21)	(0.15)	(0.19)	(0.14)
K	3.8	1.73	0.46	1.2	1.15
(SD)		(0.03)	(0.03)	(0.00)	(0.03)
P	4.1	1.51	0.43	2.1	1.21
(SD)		(0.5)	(0.09)	(0.3)	(0.14)
J	5.5	1.64	0.47	1.6	0.80
(SD)		(0.21)	(0.20)	(0.24)	(0.11)
F	5.3	2.10	0.40	1.8	0.90
(SD)		(0.12)	(0.05)	(0.81)	(0.39)

SD = Standard Deviation

values obtained lie within the normal range of 0.1 - 1.0 ml / min (Geddes & Rolla, 1988).

Volunteer (L) also showed a low stimulated flow rate. Interestingly, volunteers (K) and (L) gave the lowest mean ($\pm$ SE)Δz mineralisation rate values for the non-F paste of -96 ($\pm$ 60) and 34 ($\pm$ 60) respectively. Normal total salivary calcium concentrations range from 1.5 - 3.0 mM (Suddick, Hyde & Feller, 1980) and the slightly lower values obtained are probably due to the method adopted for determining the total concentration. Again the data collected were used to obtain further information about the volunteers and no attempt was made to correlate these variables with remineralisation rates.

The volunteers' salivary fluoride levels for the three different pastes are shown in Table 8.11. With the fluoridated pastes, the fluoride concentration in the unstimulated saliva samples for volunteers (J), (L), (P) and (F) were higher for the higher fluoride pastes. Despite the fact that paired t-tests showed no significant difference between the two fluoridated pastes, it was interesting to note that three of the four individuals who showed an increase in salivary fluoride levels with the higher fluoride paste also showed a marked increase in Δz remineralisation rate between the two pastes (Fig. 8.17). Volunteer (J) unexpectedly showed a slight decrease in remineralisation, despite the large increase in salivary fluoride levels. Only two volunteers showed a lower

Table 8.11 Stimulated (S) and unstimulated (U) salivary fluoride levels of the seven volunteers when using the three MFP pastes containing the equivalent of 0, 1000, and 2500 ppm fluoride

Volunteer	Salivary Fluoride levels (ppm)					
	non-F		1000 ppm F		2500 ppm F	
	(S)	(U)	(S)	(U)	(S)	(U)
B	0.014	0.027	0.009	0.016	0.006	0.016
D	0.014	0.033	0.012	0.014	0.012	0.012
L	0.018	0.020	0.020	0.021	0.021	0.026
K	0.014	0.027	0.013	0.020	0.018	0.020
P	0.015	0.031	0.0190	0.019	0.031	0.029
J	*	*	0.027	0.014	0.058	0.026
F	0.006	0.007	0.009	0.010	0.011	0.014

Values represent average of two readings from two different specimens.

* Volunteer did not wish to use non-F paste.

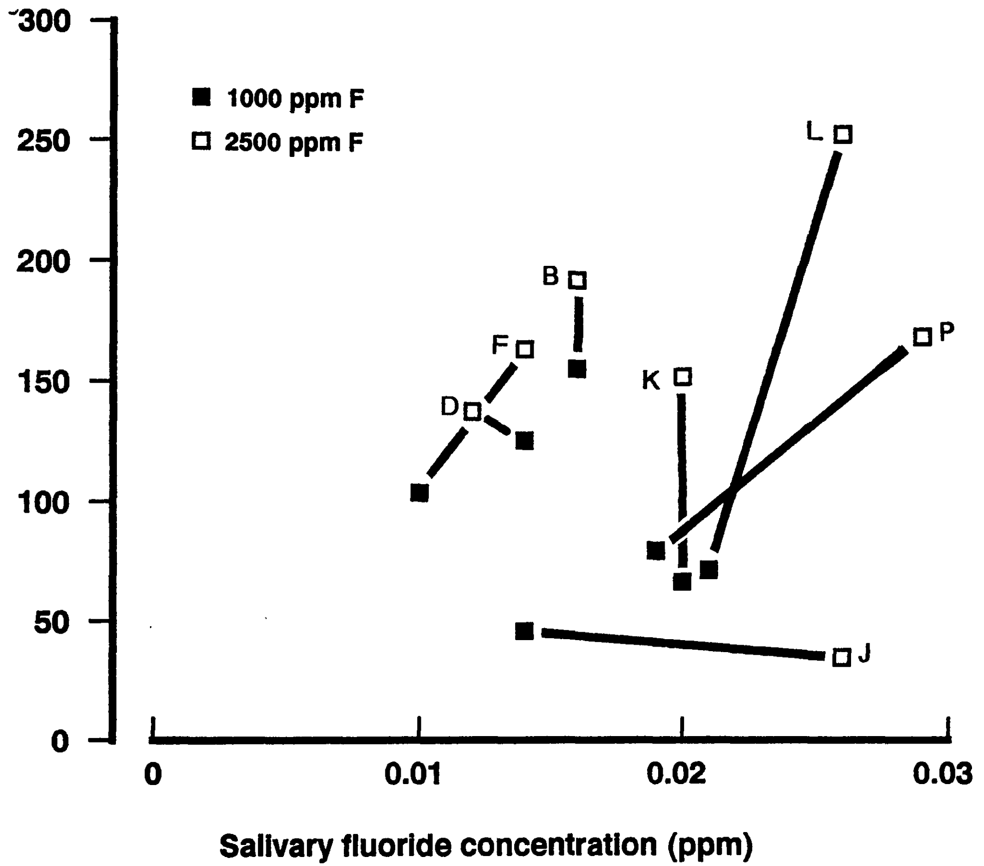


Figure 8.17 Effect of salivary fluoride concentration on Δz remineralisation rate for all volunteers using the fluoridated pastes

salivary fluoride concentration with the non-F paste compared to the fluoridated pastes. Such unexpected high fluoride values obtained with the non-F paste could be due to a carry-over effect which might have taken place with those volunteers (B), (D), (L) and (P) who used the non-F paste after the 2500 ppm fluoride paste (see Section 8.5)

8.4.3 Plaque fluoride

Due to technical difficulties, plaque fluoride analysis was performed on samples from four volunteers and only for the non-F and 2500 ppm fluoride paste (Table 8.12). Two of these volunteers (B) and (D) used the non-F paste after the 2500 ppm fluoride paste whereas the other two volunteers (F) and (K) used the control paste after the 1000 ppm fluoride paste. Plaque weights collected from the right trough were significantly greater than those collected from the left trough ($0.02>p>0.01$, paired t-test, two-tailed). No correlation was found between plaque weight and Δz remineralisation rates (Fig. 8.18). Plaque fluoride values for the 2500 ppm fluoride paste were significantly higher than those for the non-F paste ($0.05>p>0.025$, paired t-test, one-tailed). There were no significant differences in plaque fluoride levels between appliance sides. However, ignoring appliance sides it was noted that in five out of the eight possibilities (Table 8.12) an increase in Δz mineralisation rate corresponded to an increase in plaque fluoride levels.

Table 8.12 **Plaque weight and fluoride levels (ng F / mg of plaque wet weight) taken from the appliance troughs for four of the volunteers**

Volunteer	Paste	Plaque RHS weight (mg)	Plaque RHS plaque F (ng F/mg)	Plaque LHS weight (mg)	Plaque LHS plaque F (ng F/mg)
B	non-F	6.53	16.82	3.25	50.02
	2500	7.58	36.93	8.08	59.40
D	non-F	3.48	13.22	3.49	8.59
	2500	4.54	106.00	3.64	35.70
L	non-F	6.55	2.43	2.86	3.23
	2500	5.01	21.20	4.15	14.98
F	non-F	4.92	3.05	1.76	9.43
	2500	9.12	11.00	4.83	9.80

RHS = Right Hand Side
LHS = Left Hand Side

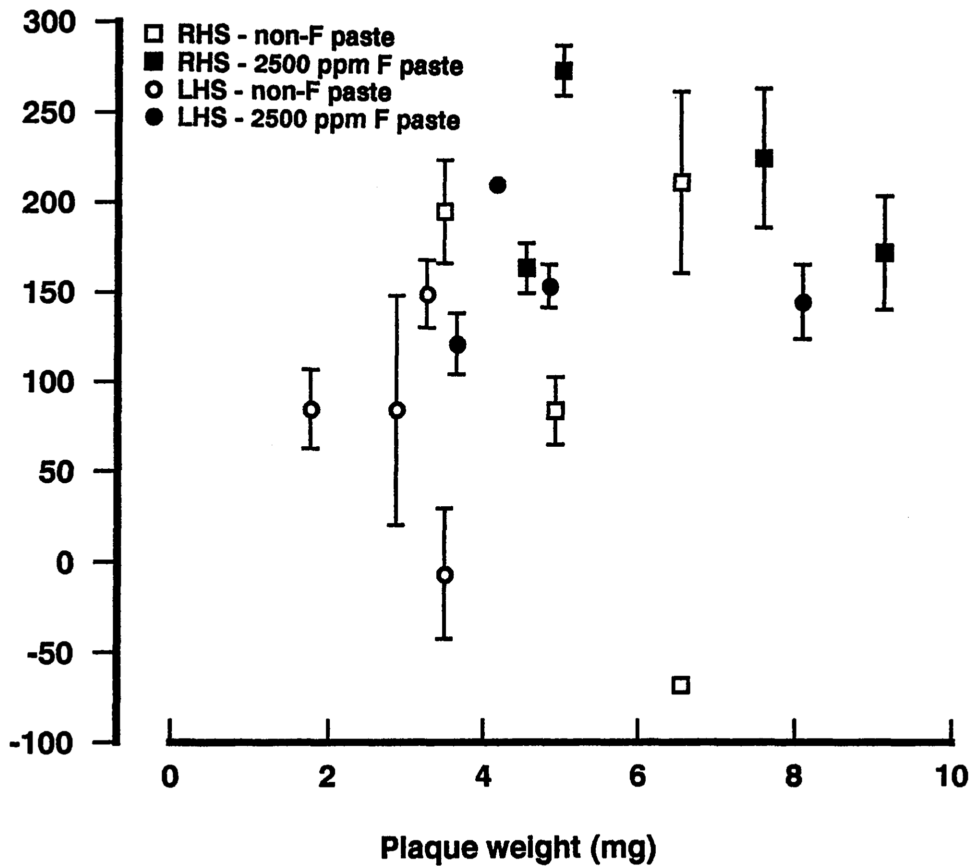


Figure 8.18 Variation in Δz mineralisation rate with plaque weight for four volunteers using the non-F and 2500 ppm F pastes
+ve values = remineralisation
-ve values = demineralisation

In Figure 8.19 the variation in Δz remineralisation rate with plaque fluoride levels is shown, using all data from both sides of the appliance and for both the non-F and 2500 pppm fluoride pastes. The Δz remineralisation rate seems to increase rapidly as the plaque fluoride levels increased to approximately 25 (ng F / mg plaque wet wt), above which there appears to be no further change in Δz remineralisation rate.

8.5 Discussion

Although numerous *in situ* studies have shown that subsurface lesions remineralise after a few weeks in the mouth, few have attempted to demonstrate a fluoride dose-response. This is probably due to the problems encountered in reducing the large variations inherent in the results from small heterogeneous groups. The improved *in situ* model described in this chapter has overcome such difficulties and successfully demonstrated significantly increased remineralisation of artificial lesions from the use of SMFP pastes containing 2500 ppm fluoride, compared to 1000 ppm fluoride. This study complements the results from a caries clinical trial (Stephen *et al.*, 1988) and a recent *in situ* study (Schafer, 1989), where the same SMFP pastes were employed. A similar relationship between increased fluoride concentration, and enhanced remineralisation, has been reported for sodium fluoride toothpastes with fluoride content levels of 300 and 1000 ppm (de Kloet *et al.*, 1986).

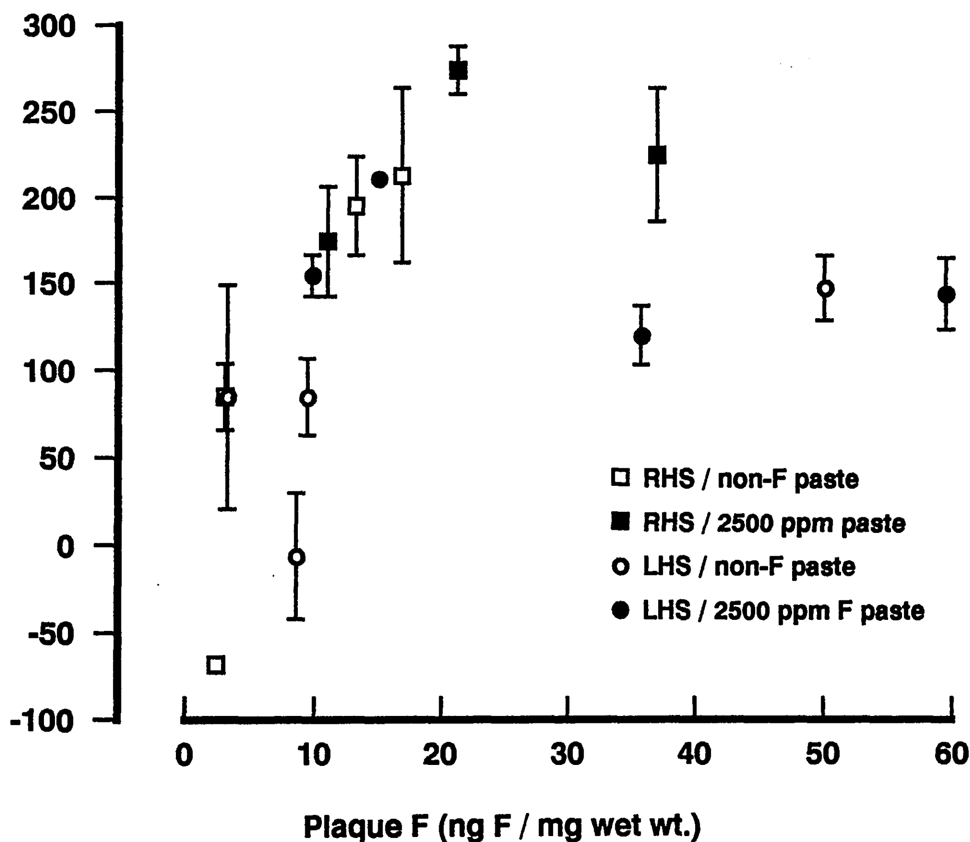


Figure 8.19 Variation in Δz mineralisation rate with plaque fluoride levels for four volunteers using the non-F and 2500 ppm F pastes
+ve values = remineralisation
-ve values = demineralisation

In the original study described by Creanor *et al.* (1987) significantly more remineralisation was observed between the non-F and 1000 ppm SMFP fluoride pastes but no differences were observed between the three fluoridated pastes i.e. 1000, 1500 and 2500 ppm. The positive results described in this chapter suggest that the sensitivity of the model was improved by the application of solution-prepared lesions. This was not surprising since as shown previously (Chapter 5 and Chapter 6), solution-prepared lesions were more responsive towards *in vitro* de-/ remineralisation processes when compared with gelatin-prepared lesions. In addition, an *in situ* study (Strang *et al.,* 1988) showed that solution-prepared lesions remineralised to a greater extent than did those which were gelatin-prepared. These changes, together with an increased number of participants, has now demonstrated that a fluoride dose-response does indeed exist.

The work reported here has shown that with these volunteers, the difference in remineralisation rate between the non-F paste and the 1000 ppm fluoride paste was not quite significant. The four volunteers who used the non-F paste after the 2500 ppm fluoride paste, gave unexpectedly high remineralisation rates for the non-F pastes. This occurred despite the two week run-in period between test pastes which is generally used in similar *in situ* studies (Schafer, 1989). It was therefore concluded that this experimental design resulted in a long-term carry-over effect, evidence of which was shown

by the high salivary fluoride levels obtained for three of the four volunteers who used the control paste after the 2500 ppm fluoride paste. This carry-over effect was an important observation and in current *in situ* studies, volunteers are now allowed a four week buffering period to ensure that such problems are minimized.

An interesting feature which emerged from this experiment was the significant difference in remineralisation found for specimens placed on either side of the appliance, with the side exhibiting greater mineralisation potential varying amongst volunteers. Large variations in mineral content changes within the same individual have also been reported in other *in situ* studies (Mellberg, Castrovince & Rotsides, 1986; Mellberg *et al.*, 1988). It is unlikely that such large variations could be attributed to inhomogeneous enamel, since sections were allocated randomly to the different sides and only those lesions with baseline Δz values lying within a narrow range were used. It is also difficult to explain clinically such site differences, because the caries activity of an individual is considered to be symmetrically distributed. Indeed the DMFS scores obtained for the volunteers (Table 8.9) showed little or no symmetrical deviation.

The variation in amount and type of plaque, as well as fluoride distribution in plaque and saliva that occurs after toothbrushing, are important factors in the caries

process. Qualitatively, the microbiological species recovered from natural and appliance plaque are similar (Creanor *et al.*, 1986b), although quantitative differences were found when the composition of appliance and natural plaque were compared. Considerable differences are also observed in the microbial composition obtained from different sites on the same tooth and at the same site on different teeth (Marsh & Martin, 1984). In retrospect, it would have been interesting to analyse the microbial composition of plaque collected from the two sides of the current appliances. Although direct correlations between certain microorganisms and demineralisation are difficult to demonstrate, studies of this kind might have contributed to an explanation regarding the observed differences.

It was found that there was a significant difference in the amount of plaque collected from the two sides (ie. right-vs-left) although there was no significant difference in plaque fluoride levels between sides. Unfortunately no measurements were made of plaque thickness which has been shown to have an effect on demineralisation *in vivo* (Wilson & Ashley, 1988; Zero, Campbell & Yang, 1988).

The significant interaction between side and volunteer showed that the difference between sides was not the same for all volunteers. Four of the seven participants gave a higher mean mineralisation rate value (for all three

parameters,(Tables 8.2 - 8.4)) for specimens on the right side for all three experimental runs, whereas the other volunteers showed no definite pattern, with the right or the left side, giving a higher rate. In order to investigate if brushing habits of the volunteers could account for such variation, subjects were questioned about brushing procedures. All except one was right-handed and first placed the brush on the upper and lower right hand side. Although, a single observation of brushing techniques cannot be considered an objective technique, it would seem that the side-effects cannot be easily explained by the demonstrated brushing methods.

In experiments of this kind, it is desirable to have a low variability between subjects since there is a better chance of detecting a treatment difference if it exists. Volunteers participating in this study were not pre-selected so that the significant variation obtained between them was not unexpected. Despite this variation, this study still showed a significant difference between the two treatment groups, thus illustrating the sensitivity of the improved model. Two-factor interactions showed that there was a significant interaction between paste and volunteer, which means that the volunteers' responses to the treatment were different. From the results it is tempting to postulate that volunteers who gave a low remineralisation rate with the 1000 ppm fluoride paste benefitted to a larger extent from the higher fluoride paste when compared with those volunteers

who had a high remineralising ability even with a low fluoride paste. Such a study is currently under way and it is hoped that the values obtained with the 1000 ppm fluoride paste will reflect the magnitude of the remineralisation values with a 2500 ppm fluoride paste.

The additional intraoral parameters reported in this thesis were performed (i) to determine the possible cariogenic potential of the volunteers, since variables such as DMFS, OHI, diet, buffer pH, calcium and fluoride levels in saliva play an important role in *in vivo* remineralisation and (ii) as part of an on-going study where such data could be used to select volunteers for their suitability to participate in similar *in situ* investigations. Although, variations in dental indices among the present volunteers existed, in general these subjects appeared to represent an average group with respect to dental motivation. As stated earlier, it was felt that there were insufficient data to correlate these parameters with de-/ remineralisation results, but it is hoped that in the future this would be feasible if the number of participants were to be increased.

In this study the relationship between remineralisation rate and salivary fluoride levels was variable. This can be explained by the small number of samples collected (three from each volunteer for each run). Salivary baseline fluoride levels are low, and close to detection with the fluoride electrode. In addition, factors such as

flow rate, time of collection and diet would also contribute to the large variation observed. By taking more frequent measurements, it would probably have been possible to demonstrate a correlation between fluoride concentration in the pastes and salivary fluoride levels as reported by Duckworth, Morgan & Burchell, 1989.

All four volunteers gave a higher plaque fluoride level with the 2500 ppm fluoride paste than with the non-F paste. The variation in Δz remineralisation rate (ignoring sides and pastes;(Fig.(8.19) gave an interesting result. It would be tempting to speculate that there was a threshold plaque fluoride level around 25 ng F / mg plaque wet weight. Below this level it may be that remineralisation rate rapidly decreases with decreasing plaque fluoride levels whereas above this value increases in plaque fluoride might have no additional effect on remineralisation. Clearly, further work is required to confirm this finding.

In conclusion, the modified appliance described here has been successful in demonstrating a fluoride dose-response (with SMFP pastes) in a natural oral enviroment. The sensitivity of this system over others described in the literature is due mainly to the combined use of thin enamel sections, where pre-and post-experimental quantification of mineral content with microradiography is possible and the appliance design which permits natural plaque stagnation without the need for microbial retaining

gauze. Such a model has many applications, already having been employed to obtain information regarding early plaque development (Macpherson, 1988), and root caries (Albashaireh, 1989). While the other observations made from study highlighted the complexity of the intra-oral environment it is hoped that, in future the undertaking of such measurements could be used to help with selection of suitable volunteers.

Chapter 9

Discussion and conclusions

9.1 Introduction

Lesion formation and mineral loss or gain have been investigated for many decades. Such studies have played a major role in understanding the mechanisms of de-/ remineralisation processes, including the beneficial effects of fluoride. The difficulties encountered in translating *in vitro* data to the complex *in vivo* situation has made *in situ* models important tools in trying to elucidate the effects of the several parameters involved. The main aims of this thesis were (i) to develop an *in vitro* pH cycling model to investigate the effect of fluoride concentration, and (ii) to study *in situ* de-/ remineralisation, particularly in relation to dentifrice fluoride concentrations.

9.2 Enamel inhomogeneity

One of the problems encountered in de-/ remineralisation studies is the susceptibility of the tissue to a cariogenic challenge. Most *in vitro* and *in situ* investigations reported have tried to overcome this difficulty by choosing a control specimen from the same human tooth, by using bovine enamel which is a more homogeneous material or by abrading the enamel surface. These techniques offer little improvement since, (i)

large variations in enamel are present, even within the same tooth as in going from the incisial to the cervical margin (Poole, Newman & Dibdin, 1981; ten Cate *et al.*, 1988), (ii) bovine and other non-human enamel is more porous and demineralises faster than human enamel (Shellis & Tyler, 1981) and (iii) abrasion, although it removes the high surface fluoride levels, also produces a porous material and most importantly does not completely eliminate enamel inhomogeneity. In Chapter 3, it was shown that the demineralisation of bulk and thin sections of enamel were identical. This result enables the use of single sections of human enamel in de-/ remineralisation studies, with the consequent advantage that mineral content can be quantified repeated ly, thus overcoming enamel inhomogeneity problems. In addition, only premolar teeth, extracted for orthodontic reasons were used. Any inherent fluoride present in the enamel material was not detected in any of the *in vitro* demineralisation solutions used throughout this study.

9.3 pH cycling

pH cycling models have been employed to study preventive treatments incorporating fluoride and also to obtain information regarding fluoride dose regimens. In order to obtain realistic pH cycling conditions, the preliminary studies described in Chapter 4 were carried out and compared to a previous *in situ* study where the demineralisation rate of sound enamel sections was pursued.

Gelatin-prepared lesions were cycled daily using calcium phosphate solutions of varying hydrogen ion concentrations. Decreasing the pH resulted in increased demineralisation of the enamel specimens. However, Margolis, Murphy & Moreno (1985) showed that the driving force for enamel demineralisation is best described by the degree of saturation of the demineralisation medium with respect to enamel, and not by simpler parameters such as pH. Therefore, more information might have been obtained if the concentrations of calcium and phosphate ions were also varied. Nonetheless, the study demonstrated the ease with which demineralisation of varying magnitudes could be obtained with aqueous organic acids. These have the advantage over the gelatin-system in that they can be chemically well-defined.

The timing of the de-/ remineralisation cycles was empirically based, and 16 hours' demineralisation and 8 hours' remineralisation proved to be too drastic. Therefore, as shown in Chapter 6, the daily demineralisation cycle was reduced to three hours. The *in vitro* studies described in this thesis represent a simple application of the pH cycling method and like most models did not include the effects of salivary proteins, pellicle and plaque on diffusion and mineralisation inhibition. In addition, cycling times were not representative of the oral situation where both processes take place almost simultaneously. Despite the obvious shortcomings, the model was successful in demonstrating

the beneficial effects of the continual presence of fluoride, as well as showing a fluoride dose-response with neutral sodium fluoride solutions (Chapter 7). pH cycling models, therefore, have an important role in the preliminary assessment of factors which may influence remineralisation.

9.4 Artificial lesions

To date there is still no generally accepted theory to explain the phenomenon of subsurface demineralisation. Despite this, it is very simple to produce carious enamel *in vitro* and numerous methods have been employed, each capable of producing lesions, which are histologically similar to natural ones (Margolis & Moreno, 1990). It is generally agreed that natural lesions are not suitable for studying de-/ remineralisation processes since it is not easy to determine whether these are active or "arrested". As discussed in Chapter 5, it has been suggested that the way artificial lesions are prepared may influence their behaviour in subsequent studies (Mellberg & Chomicki, 1983). This was demonstrated in the comparative study described in Chapter 5 where it was concluded that solution-prepared lesions were more responsive towards de-/ remineralisation challenges. This finding was complemented by the pH cycling study (Chapter 6) where the effect of the continual presence of low levels of fluoride was investigated. This study indicated that solution-prepared lesions were superior to gelatin-prepared lesions when studying fluoride efficacy

since the former lesions were more responsive, more standarized and were not contaminated with fluoride. Such findings were relevant to the *in situ* study presented in Chapter 8.

9.5 In situ studies

In situ studies provide a natural environment where de-/ remineralisation takes place and can give potential information regarding several other parameters known to be involved in the caries process. The most widely used model makes use of removable appliances that rely on the use of dacron gauze (Koulourides *et al.,* 1974), the presence of orthodontic bands (Ogaard *et al.,* 1986) or the use of a plaque retentive-area (Creanor *et al.,* 1986a; Wefel *et al.,* 1987).

It is not easy to conclude what has made the *in situ* model used in this thesis more successful than the original version used by Creanor *et al.* (1987). However, the *in vitro* studies described in this thesis, together with the comparative *in situ* remineralisation study reported by Strang *et al.* (1988), strongly suggest that the method of lesion preparation (ie. the use of solution-prepared lesions) has contributed significantly to the sensitivity of the model.

The interactions observed in the *in situ* study were not unexpected since many factors are known to be involved in *in situ* de-/ remineralisation. The

additional measurements (Section 8.2.7) carried out on the volunteers gave speculative results. However, as mentioned previously, there were insufficient data to draw any conclusions. Nonetheless, it is hoped that in future more data will be collected, in order to (i) correlate single parameters with *in situ* remineralisation, and (ii) use such data to screen volunteers prior to enrolling them in similar *in situ* studies.

As mentioned in Section 9.1, it is difficult to extrapolate *in vitro* results to the *in vivo* situation, because additional factors such as the effect of plaque, dilution by saliva, fluoride availability from the product and clearance from the oral cavity, have to be taken into account. However, if one assumes that the initial dilution of a dentifrice in saliva is of the order of 1:3 to 1:5, then it is not unrealistic to suggest that the fluoride concentration of 500 ppm which gave maximium *in vitro* remineralisation (Chapter 7) is equivalent to the fluoride concentration available in the 2500 paste used in the *in situ* study. Further information regarding maximium fluoride doses *in situ* could be obtained by using dentifrices with a higher concentration than 2500 ppm, if the problem of potential fluoride toxicity could be overcome.

9.6 Conclusion

Work reported in this thesis gives further evidence that *in vitro* pH cycling and *in situ* studies are

potential tools in the study of de-/ remineralisation of dental enamel. pH cycling provides a simple method for investigating fundamental relationships (eg. effect of fluoride concentration on remineralisation), whereas the *in situ* studies give information on how the complex oral environment modifies such relationships.

Appendix I - Materials

Disposal materials

Universal containers were supplied by Gibco (Europe) Ltd., Paisley.

Microwells were obtained from Orion Research Incorporated Ltd., Massachusetts, USA.

Plastic beakers were supplied by BDH, Chemicals Ltd., Poole.

Pipettes and bijou bottles were supplied by Sterlin Ltd., Middlesex.

Eppendorf tubes (1 mL capacity) were supplied by Anderman and Co., East Molesey.

Sterile plastic syringes were obtained from Becton Dickinson, Dublin.

Glass microscopic slides and coverslips were obtained from Chance Propper Ltd., Warley.

Screw cap containers (200 mL) were supplied by Inverclyde Sciences, Greenock.

Micro tubes were supplied by Salstedt, Leicester.

Photograpic material

Microradiographic plates (Kodak high resolution plates Type 1A) were manufactured by Eastman Kodak Company., Rochester, New York, USA.

Developer (D-19), clearing solution (Dacomatic DN3 / DR-5) and rapid fixer were supplied by Kodak Ltd., Hemel, Hampstead.

Chemicals

Calcium chloride, sodium dihydrogen orthophosphate, glacial acetic acid, sodium hydroxide, potassium hydroxide, sodium chloride, sodium fluoride, thymol, silicon carbide (superfine, 600 grid), ammonia, solochrome black, nitric acid, hydrochloric acid, perchloric acid, quinoline, methyl alcohol, acetone, ethylene diaminetetraacetic acid (EDTA), magnesium sulphate heptahydrate, were all of analytical grade and were supplied by B.D.H. Chemicals Ltd., Poole.

Appendix I (continued)

Trans -1,2- diaminocyclohexanetetraacetic acid (CDTA) was supplied by Sigma Chemical Company, Ltd.,Poole.

Gelatin powder was obtained from Difco Laboratories, Surrey.

Amyl acetate and alcohol were obtained from May and Baker Ltd., Dagenham.

Total Ionic Strength Adjustor (TISAB III) and sodium fluoride standard solution (100 ppm) were obtained from Orion Research Incorporated Ltd., Massachusetts, USA.

White bauxlite 1200 was manufactured by Raymond A. Lamb, London.

Miscellaneous materials

Cling film, aluminium foil, acid resistant nail varnish (Max Factor, London), super glue (Lockite, Welywn Garden city) were obtained from a retail shop in Glasgow.

Discs for dental hand piece were supplied by Chaperlin and Jacobs Ltd., Surrey.

Scapel blades were supplied by Swann Morton Ltd., Sheffield.

Adhesive tape was manufactured by Electronic, Mecanorma, France.

Paraffin wax was obtained from Orion Diagnostica Helinski, Finland.

Appendix II - Instructions to volunteers

1. The appliance should be worn at all times except where detailed below.

2. Brush TWICE per day, (morning and night) with the coded dentifrices and toothbrush provided.
(a) Remove the appliance. You may clean the appliance but do not disturb the trough area with the experimental sections. With the appliance out of the mouth clean the lower lingual aspects of your teeth with brush and water. At this stage the teeth may be flossed etc.

(b) Insert the appliance. Extrude a half inch of toothpaste on to the brush and with the appliance in place clean all other areas for two minutes.

(c) Spit out excess paste but DO NOT RINSE OUT.

3. You should not use any other dental fluoride products. However, if you feel that you must clean your teeth at other times, we will supply you with a non-F paste. Use a separate brush and remove the appliance while you brush your teeth. Rinse out thoroughly before replacing the appliance.

Appendix III - Food intake questionaire

Name: Date:

Age: Height: Weight:

1. Please record in detail everything you eat or drink in the order in which it is eaten.
2. Include not only meals but between-meal snacks.
3. Use household measurements such as 1 serving, 1/2 cup, 1 teaspoon.

Day	b/fast	between meals	lunch	between meals	dinner	evening
1						
2						
3						

Appendix IV - Protocol compliance questionnaire

Dear Volunteer,

I should be grateful if you would answer the following questions.
Prior to your involvement in the in situ study:

---

1. How many times per day did you clean your teeth with fluoride dentifrice?

---

2. Did you always use a specific dentifrice? If so, give the trade name.

---

3. Did you regularly use any other fluoride suppleme mt? For example. mouthrinse, gels etc. Please give details.

---

4. Did you have any dental treatment when you were wearing the intraoral appliance?

---

5. Was wearing the appliance uncomfortable, painful, a nuisance, no bother?

---

6. Did you regularly remove your appliance before or after food intake?

---

7. How many times do you normally rinse your mouth after brushing? Once, twice, three times, more?

---

8. Did you often miss brushing your teeth with the dentifrice provided?

---

9. Do you remember the code of the dentifrice you used last?

---

10. Would you consider taking part in another study using the intraoral appliance?

Thank you for your cooperation.

Appendix V - Analysis of variance of data from Chapter 8

Δz mineralisation rate

variation	Sum of squares	d.f.	mean square	F-ratio	Sign. level
main effects	837534	9	93059	14.192	0.0000
paste	258732	2	129366	19.728	0.0000
volunteer	471053	6	78508	11.973	0.0000
side	155845	1	155845	23.767	0.0000
Two-factor interactions	480300	19	25278	3.855	0.0000
paste / volunteer	281356	11	25577	3.901	0.0001
paste / side	22514	2	11257	1.717	0.1837
volunteer / side	181588	6	30264	4.615	0.0003
Residual	845896	129	6557		
Total (corr.)	2163730	157			

Appendix V (continued)

surface zone (SZ) mineralisation rate

variation	Sum of squares	d.f.	mean square	F-ratio	Sign. level
main effects	95.124	9	10.568	10.150	0.0000
paste	43.707	2	21.853	20.988	0.0000
volunteer	53.278	6	8.878	8.527	0.0000
side	10.185	1	10.184	9.782	0.0022
Two-factor interactions	32.904	19	1.731	1.663	0.0509
paste / volunteer	21.522	11	1.956	1.879	0.0478
paste / side	0.597	2	0.298	0.287	0.7512
volunteer / side	9.400	6	1.566	1.505	0.1816
Residual	134.320	129	1.041		
Total (corr.)	262.346	157			

Appendix V (continued)

lesion body (LB) mineralisation rate

variation	Sum of squares	d.f.	mean square	F-ratio	Sign. level
main effects	148.6	9	16.52	19.68	0.0000
paste	20.1	2	10.06	11.99	0.0000
volunteer	117.5	6	19.59	23.33	0.0000
side	18.8	1	18.81	22.41	0.0000
Two-factor interactions	64.1	19	3.37	4.01	0.0000
paste / volunteer	53.8	11	4.8	5.83	0.0000
paste / side	0.49	2	0.25	0.30	0.7430
volunteer / side	14.55	6	2.42	2.88	0.0113
Residual	108.28	129	0.839		
Total (corr.)	321.03	157			

List of Publications

The following papers have been published, or accepted for publication, from the work associated with this thesis:

1. Strang, R., Damato, F.A., Creanor, S.L. and Stephen, K.W. (1987) The effect of baseline lesion mineral loss on *in situ* remineralisation.
   Journal of Dental Research, 66, 644 - 1646

2. Damato, F.A., Strang, R., and Stephen, K.W. (1988) Comparison of solution- and gel-prepared enamel lesions - an *in vitro* pH-cycling study.
   Journal of Dental Research, 67, 1122 - 1125

3. Strang, R., Damato, F.A., and Stephen, K.W. (1988) Comparison of *in vitro* demineralisation of enamel sections and slabs.
   Caries Research, 22, 348 - 349

4. Damato, F.A., Strang, R. and Stephen, K.W. (1990) Effect of fluoride concentration on remineralization of carious enamel: an *in vitro* pH cycling study.
   Caries Research, In press

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