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REMINERALISATION OF THE INCIPIENT ENAMEL LESION

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PUBLICATIONS

The following are (a) published papers by the author relating to the work of this thesis, (b) papers presented by the author and (c) in association with the author at Scientific meetings related to the work contained in this thesis.

(a) CREANOR, S.L., STRANG, R., TELFER, S., MACDONALD, I., SMITH, M.J., and STEPHEN, K.W. (1986)
In situ appliance for the investigation of enamel de- and remineralisation. A pilot study.
Caries Research, **20**, 385 - 391.

CREANOR, S.L., MACFARLANE, T.W., MACKENZIE, D., WEETMAN, STRANG, R. and STEPHEN, K.W. (1986)
Microbiology and acid/anion profiles of enamel surface plaque from an in situ caries appliance.
Caries Research, **20**, 392 - 397.

(b) CREANOR, S.L., WEETMAN, D., STEPHEN, K.W., TELFER, S., STRANG, R. and BURCHELL, C.K. (1984)
A microbiological assessment of an intra-oral appliance for in situ caries studies.
Journal of Dental Research, **63**, 497. (Paper presented at BSDR meeting, London, 1984)

CREANOR, S.L., TELFER, S., SMITH, M.J., STEPHEN, K.W., STRANG, R. and BURCHELL, C. (1985)
In situ caries studies using an intra-oral appliance.
Caries Research, **19**, 178. (Paper presented at 31st ORCA meeting, Noordwijkerhout, The Netherlands, 1984)

CREANOR, S.L., STRANG,R. and STEPHEN, K.W. (1986)
Remineralisation of artificial lesions in situ.
Caries Research, **20**, 175. (Paper presented at 32nd ORCA meeting, Stenungsund, Sweden, 1985)

CREANOR, S.L. (1986)
An investigation of enamel remineralisation using an in situ appliance.
Journal of Dental Research, **65**, 488. (Paper presented at BSDR meeting, Dundee, 1986)

CREANOR, S.L., STRANG, R., DAMATO, F.A., MACDONALD, I. and STEPHEN, K.W. (1987)
A cross-over study of artificial lesion remineralisation in situ.
Caries Research, **21**, 173. (Paper presented at 33rd ORCA meeting, Lofthus, Norway, 1986)

(c) STRANG, R., MACDONALD, I., CREANOR, S.L. and STEPHEN, K.W. (1986)
Mineral content variations in demineralised enamel. Caries Research, 20, 185. (Paper presented at 32nd ORCA meeting, Stenungsund, Sweden, 1985)

MACDONALD, I., CREANOR, S.L., STEPHEN, K.W. and STRANG, R. (1986)
Comparison of _in situ_ and _in vitro_ enamel demineralisation rates.
Caries Research, **20**, 167. (Paper presented at 32nd ORCA meeting, Stenungsund, Sweden, 1985)

WISHART, N.F., CREANOR, S.L., MACDONALD, I., DAMATO, F.A., STRANG, R. and STEPHEN, K.W. (1986)
A pilot study of an _in vitro_ model for the investigation of remineralisation.
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DECLARATION

This thesis is the work of the author, and associates under the direct supervision of the author.

SUMMARY

The anticaries activity of the fluoride ion has been recognised for many years. The precise mechanism of action is not clear, although in recent years fluoride is thought to influence the natural remineralising properties of saliva on porous enamel. However, there is confusion as to the most effective means of delivery, and parameters such as concentration, duration and frequency of exposure to fluoride have been empirical.

The primary aim of the work contained in this thesis, therefore, was to develop and validate a means by which remineralisation of early enamel lesions within the oral environment, could be quantitatively assessed. Thus, an *in situ* appliance was designed on which sections of enamel could be mounted and removed at intervals, for mineral content measurements to be carried out using microradiography and microdensitometry. The use of single sections, necessitating no other control tissue, made a major contribution to the sensitivity of mineral content changes assessed throughout the duration of this project. Validation of the technique showed conclusively that the artificial stagnation area had fundamental similarities to the environment of a natural contact point.

The major part of the work was concerned with an assessment of remineralisation in early enamel lesions, when exposed to differing concentrations of dentifrice fluoride, when used by volunteers wearing *in situ* devices.

The results indicated that in a small panel of dentally-orientated subjects, the maximum remineralising potential of a fluoridated dentifrice had been attained by 1000 ppmF, the lowest concentration investigated. Whilst being significantly better than the non-fluoridated placebo, in general no significant differences were detected between different fluoride concentrations. Nevertheless, in agreement with other workers, some person-to-person variation was detected, especially when analyses of the fluoridated dentifrices was performed.

In an attempt to develop a laboratory test to mimic the *in situ* results, an *in vitro* model was proposed. Whilst the model failed to detect differences in remineralising abilities of the fluoridated dentifrices, no significant differences were detected between those and the non-fluoridated placebo.

In conclusion, the *in situ* device would appear to have successfully linked the advantages of the single section technique and the natural oral environment. Of further interest would be changes to the parameters investigated in this work: such as lowering of the fluoride content, the duration and the frequency of exposure to the fluoridated dentifrices. Further work into both the microbiological and biochemical changes in the plaque overlying the enamel lesion would be of considerable interest, as well as extending the technique to involve dentine caries, both coronal and root.

CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION AND AIMS

There have been few public health measures which have been studied so intensively over so long a period and under such a variety of conditions, as the caries preventive effect of fluoride. Nevertheless, the mode of action of fluoride has not yet been explained fully. Clinically, in temperate climatic areas where water is naturally fluoridated at substantially less than the "optimal" 1 ppm level, and where artificial water fluoridation is not a viable proposition, a number of vehicles have been used as sources of fluoride. These have included drops and tablets for preeruptive use, and tablets, dentifrices, gels, mouthrinses etc. for topical, posteruptive exposure. Table 1.1 outlines the range of concentrations and frequencies which have been used as part of typical caries prevention programmes. The rationale supporting the advised concentrations, frequencies and durations of exposure to many of these modalities is, however, entirely empirical. Unfortunately, to test adequately any one vehicle, its mode of delivery and associated parameters, would normally require a three-year, double-blind clinical trial.

One of the objectives of this thesis, therefore, is to document a technique which will help solve some of the problems and assist in rationalising the topical use of

fluoride.

Thus, the aims of the work were:

1. to design and develop a new *in situ* caries-investigative method,

2. to carry out an *in situ* remineralisation study using dentifrices of differing fluoride concentrations,

- and -

3. to develop an *in vitro* model to simulate some of the conditions of the enamel/plaque interface zone.

In this chapter, a discussion of the carious process will be presented, along with a review of the literature with regard to the proposed caries-inhibiting mechanisms of fluoride. In addition, the advantages and disadvantages of previous *in vivo*, *in situ* and *in vitro* techniques and of the various methods of lesion assessment will be discussed.

1.2 THE CARIOUS PROCESS

Dental caries can been described as the progressive dissolution of the mineral component of the dental hard tissues, resulting in structural breakdown and eventual cavitation. It is normally at this stage that intervention by the dental surgeon is indicated, to prevent further spread of the disease and consequent loss of the tooth.

The earliest macroscopic evidence of the carious process is a small opaque white region, the "white spot". This lesion, which cannot be detected at contact points, is an area of increased porosity and will not normally be detected on routine bitewing radiographs (Silverstone, 1977). It has been suggested that the stage of lesion progression before cavitation, is the state most susceptible to redeposition of mineral within the spaces of the "white spot" lesion. This process has been termed "remineralisation". An example of such a lesion is demonstrated in Fig. 1.1, although here, part of the "white spot" has already broken down, resulting in cavitation.

A constant exchange of calcium and phosphate ions between enamel and saliva has been postulated. This balanced situation is dependent on a number of local factors, such as the presence of plaque and the availability of refined carbohydrates, such as sucrose. If loss of mineral predominates, (a consequence of increased bacterial acid production), there will be progressive loss of calcium, resulting in a calcium-deficient mineral as compared to the surrounding enamel (Arends & Davidson, 1975; Ingram & Silverstone, 1981). If this depletion is allowed to continue, there will be complete dissolution of the mineral phase, loss of structural integrity and invasion of the lesion by oral bacteria (Brannstrom _et al._, 1980). Following cavitation, the bacteria can colonise the defect easily and establish an ecosystem. This results in further progression of the carious process

into the deeper parts of the enamel and eventually the dentine, a consequence of continued bacterial production of organic acids.

If remineralisation is the predominant process, then the sequence of events discussed above, will, in general, be reversed. Remineralisation will be enhanced in the presence of fluoride (Koulourides et al., 1961) and it was the influence of fluoride concentration on this process which was investigated in this project.

1.3 FLUORIDE AND ITS ANTICARIES MECHANISMS

1.3.1 Introduction

Fluorine is a highly reactive halogen. Its negatively charged ion, although much less reactive, has an important role in successful remineralisation of early enamel carious lesions.

The anticaries activity of the fluoride ion has been known for many years. However, the mode of action of fluoride has still not been explained fully. The following sections will deal with the historical aspects of the use of fluoride as a caries preventive measure and will consider some of the evidence supporting its use. In addition, the variety of techniques which have been proposed to deliver fluoride to the oral environment will be discussed.

1.3.2 Historical Account

An association between enamel mottling and the environment was noted independently in Naples and in Colorado. Eager (1902), observed the presence of white and brown stains in persons who had been born and resided in Naples. He made the unfounded conjecture that an agent, associated with the nearby volcano, Mount Vesuvius, was transported to the population via the water supply, and was presumably responsible for the clinical lesions. In parallel, McKay (1916) noticed similar enamel changes in Colorado residents and gave this condition the term "mottled enamel".

In 1916, McKay claimed that the causative factor of "mottled enamel" was present in the water supply consumed during the period of tooth mineralisation. In addition, reporting on the appearance of mottled enamel in Britton and Oakley, McKay (1925) linked the mottling to the water supplies, which were recommended to be changed. This conclusion was supported by the absence of mottling in children born after the water supplies to the towns were subsequently altered (McKay, 1933). Further evidence supporting the association between enamel mottling and water supplies was obtained in Bauxite, where mottled enamel also became apparent several years after a change in the water supply. Initial analyses of the water did not indicate a probable causal agent (Kempf & McKay, 1930). However, Churchill (1931) who made a specific

analysis for trace elements, found a relatively high concentration of fluoride (13.7 ppmF). Consequently, an analysis was made of waters from the areas which McKay had previously described as endemic regions of enamel mottling and all were shown to contain fluoride in concentrations above 2.0 ppm. Fluoride, it was postulated, was the causative agent of mottled enamel (Churchill, 1931).

The association between mottled enamel and caries reduction was discussed in a number of reports (eg. McKay, 1916; Bunting et al., 1928). However, it was not until the observations of Ainsworth (1928, 1931), that the association between decreased caries prevalence and high water fluoridation was recognised.

As a result of the above, the U.S. Public Health Department commissioned Dean (1933, 1934 & 1936) to discover the extent and geographical distribution of mottled enamel in the United States (The "Shoe Leather" Survey). Dean established that in those areas where the water supply had concentrations of around 1.0 ppmF the inhabitants had minimal enamel mottling and a significantly lower caries prevalence. This survey paved the way for a much larger investigation of caries experience in 7257 children (aged 12 - 14 years) who had used their local water supplies from birth. Water fluoride concentrations ranged from zero to 2.6 ppm, and Dean's new data confirmed earlier findings, relating to the benefits of water fluoridation from birth. Although complete inhibition of caries did not occur, a 60 per cent

difference was observed between the areas with > 1.0 ppmF in their water supplies and the low fluoride towns. In addition, it was noted that in those who moved into naturally fluoridated areas after the age at which coronal calcification was complete, no such mottling was detected. Hence, high concentrations of fluoride either ingested or applied topically, could not cause fluorosis by this stage of development. Dean et al. (1942) concluded that a concentration of fluoride at 1.0 ppm in the drinking water showed near maximum caries reduction, and caused only "sporadic instances of the mildest forms of dental fluorosis of no practical aesthetic significance".

The main conclusions from the extensive data regarding the relationship between caries prevalence and the concentration of fluoride in the drinking water were that:

1. natural water fluoridation at a level of 1.0 ppm produced a striking decrease in caries with no fluorosis of aesthetic significance;
2. fluoride at the 1.0 ppm level in the water must be ingested during the pre-eruptive stages of dental development to gain maximum benefit in caries reduction;
3. there was less benefit, although detectable, if the water fluoride level was less than 0.5 ppm;
4. while some additional caries-inhibiting benefit was observed above a fluoride level of 1.0 ppm, unacceptable fluorosis occurred at levels over 1.5 ppmF.

Following Dean's epidemiological observations, the addition of fluoride to a less than optimally fluoridated water supply was proposed. The first such study was initiated at Grand Rapids, Michigan, in the mid-1940's. It was clearly established six years later that the artificial addition of 1.0 ppmF had the dramatic 50 per cent caries reduction effect expected, and that this occurred mainly in those individuals who had been born and brought up in the area, throughout the period of water fluoride adjustment (Arnold et al., 1953).

Until 1946, it had been assumed that enamel would only benefit if fluoride exposure was available during the preeruptive developmental stages. However, in that year, Klein (1946) examined Japanese children transferred from an area of low fluoride (0.1 ppm) to Arizona, where the water supply contained approximately 3.0 ppmF, and showed that teeth in the process of eruption also benefitted. In addition, teeth already erupted benefitted, albeit to a lesser extent. Clearly a topical effect must have occurred since the erupted enamel could not have been altered otherwise. In addition, a study in naturally fluoridated Boulder, showed that the benefits of fluoride exposure could be continued into adult life (Klein, 1946). Thus, the value of fluoride applied topically now became apparent. While others concentrated on the water fluoridation aspects, as early as 1941, Bibby et al. (1946) initiated the first ever topical fluoride study. This was followed by Roberts et al. (1948), and although

neither study was particularly successful, apropos demonstrating a caries reduction effect in teeth on which the vehicle had been applied, it is interesting to note that even in these early years, alternative means of delivering fluoride, other than water fluoridation, were being investigated.

1.3.3 Fluoride mechanisms - General

As discussed above, fluoride is thought to have both a systemic and a topical role. Fluoride is acquired into enamel by the systemic route from the ingestion of fluorides in water, beverages, foods or supplements, and topically from oral fluids and topical fluoride preparations. While, topical acquisition is restricted to the enamel surface, systemic fluorides are predominantly incorporated preeruptively during the process of maturation.

The proposed mechanisms of the cariostatic action of fluoride are thought to involve several subtle effects on the mineral phase of the enamel and the metabolism of the dental plaque bacteria:

(a) during initial mineralisation of the developing tooth fluoride mediates the transformation from the soluble calcium-phosphate phases to the more stable, less soluble hydroxyapatite and fluoridated hydroxyapatite (Brown et al., 1977; Amjad & Nancollas, 1979).

(b) plaque fluoride, particularly during acid attacks or after topical application of fluoride, may reach ionic

concentrations high enough to inhibit bacterial metabolism (Hamilton, 1977). However, Carlsson et al. (1969) had previously shown that the bacterial enzymes which produce glucan and fructan from sucrose are unaffected.

(c) it has been postulated that fluoride may have a preeruptive effect on the cusp, pit and fissure morphology of the tooth, by reducing the depths of the fissures (Jenkins, 1963, 1970). This mechanism alone, however, would not account for the observed reduction in smooth surface caries.

(d) the mechanism, now thought to be of greatest importance, which in many ways is not dissimilar to (a) above, is fluoride's influence on the remineralisation process (ten Cate & Duijsters, 1983 a,b). It is proposed that the presence of fluoride produces larger, less reactive and more acid-resistant crystals which fill the spaces produced by the demineralisation process (Silverstone, 1977).

1.3.4 The systemic role of fluoride

Fluoride from the systemic circulation is incorporated into the enamel during its maturation, before exposure to the oral environment, and is thought to originate from the tissue fluid surrounding the dental papilla. The systemic mode of action has been attributed to the acid-resistant properties of fluoride-containing crystallites (Frazier et al., 1967). Fluoride can be considered as a contaminant and although the fluoride concentration is relatively high within the maturing enamel, this decreases as more mineral

is being deposited (Weatherell *et al*., 1977). The mineral component of enamel is made up mainly of calcium, phosphate, carbonate and hydroxyl ions, and because of the presence of trace elements such as fluoride, chloride, strontium, sodium and magnesium, which may substitute for either the calcium or the phosphate, it does not exhibit the same calcium to phosphate ratio as the stoichiometric hydroxyapatite. The addition of other elements generally increases the solubility. The substitution of a fluoride ion for a hydroxyl group, however, greatly reduces the enamel solubility, even when only 10 per cent of such F/OH substitutions occur as with systemically fluoridated enamel (Fejerskov *et al*., 1981). The addition of the fluoride ion to the hydroxyapatite alters both the physical and chemical properties of the crystals (Neuman *et al*., 1950, Ingram & Nash, 1980).

1.3.5 Topical fluoride and its role in remineralisation

Whilst the evidence supporting the systemic role of fluoride is overwhelming (section 1.3.2), there is considerable evidence that topical application of fluoride can have a profound effect, and it is now well established that mature, erupted enamel can be altered from topical exposure to fluoride. Head (1912) concluded that artificially softened enamel would reharden after immersion in natural saliva. More recently it has been shown that a considerable degree of rehardening of presoftened enamel may be achieved solely by exposure to saliva (Koulourides *et al*., 1961; Pigman *et al*., 1964;

Koulourides *et al*., 1965; Pickel *et al*., 1965; Feagin *et al*., 1969). Fluoride, however, has been shown to increase both the hardness and acid resistance of porous enamel (Koulourides *et al*., 1961). By diffusing into enamel, fluoride is purported to encourage the formation of fluorapatite - a more acid resistant relative of hydroxyapatite (Mellberg *et al*., 1966; Kirkegaard, 1977 a,b; ten Cate & Duijsters, 1983 a,b).

In vitro evidence has shown enhancement of the natural remineralisation process by the addition of fluoride (Silverstone *et al*., 1981; Featherstone *et al*., 1982). Koulourides *et al*. (1961) have shown as much as an eight times increase in the rate of remineralisation *in vitro* by the addition of 0.05 mM fluoride to a calcium phosphate solution.

Clinical evidence of early enamel lesion remineralisation has been documented (von der Fehr *et al*., 1970; Grondahl, 1979). However, although over 50 per cent of white spot lesions created in the study of von der Fehr *et al*. (1970) disappeared, the clinical appearance of others persisted. It is important to remember, however, that the presence of such a white spot need not imply there is active caries, as it may not be necessary to remineralise the entire lesion for the enamel to become protected (Briner *et al*., 1974; Silverstone *et al*., 1981). If the surface becomes remineralised, as occurs in exposure to high concentrations of fluoride and calcium, then the underlying enamel lesion remains unchanged. Thus, the visual appearance of the white spot lesion persists but the lesion would be considered arrested and certainly

resistant to further acid attack (ten Cate & Duijsters, 1982).

Topical acquisition of fluoride on to the enamel surface normally results from contact with water, food, beverages and fluoridated dentifrices, rinses, gels, varnishes, solutions etc. Fluoride appears to be preferentially taken up by areas of increased porosity such as etched enamel or the early enamel lesion (Joyston-Bechal & Kidd, 1980). In high concentrations of fluoride, such as those found in gels, mouthrinses and dentifrices, the fluoride ion is thought not only to exchange with the apatitic hydroxyl group, but also form calcium fluoride on the enamel surface. Since this is a relatively unstable compound, most of it breaks down and diffuses back into saliva. However, a small amount is thought to remain within the pores of the enamel lesion and act as a nidus of fluoride (Mellberg *et al*., 1966; Kirkegaard, 1977a; ten Cate & Duijsters, 1982).

1.3.6 Fluoride vehicles

As a consequence of the evidence supporting the topical role of fluoride (Klein, 1946; Ast & Chase, 1953) and, since all domestic water supplies world-wide do not contain an effective level of fluoride, the commercial production of alternative vehicles has received a great deal of attention. In addition, as water fluoridation schemes demand not only a reliable mains water supply, but also an equally reliable grid electricity system for

equipment control, other means of community-based fluoridation have been suggested and implemented. Thus, when water fluoridation is not available during preeruptive enamel maturation, drops and tablets can provide a practical alternative, but demand strict cooperation to maximise the benefit. Currently, there is a continuing discussion regarding the exact preeruptive dose. Nonetheless, any dosage regime has to be adjusted to account for the varying quantities of fluoride which are always present, even at trace levels, in any domestic water supply.

Salt has been proposed as an alternative means of both systemic and topical fluoridation (Wespi, 1948; WHO, 1976; Toth, 1976), and has been introduced in Switzerland, Columbia, and Hungary, at a level of 350 mg F per Kg in the latter country. Although caries reductions as great as 60 per cent have been reported in trials carried out (Toth, 1976), interest has diminished in most western countries with the advent of more controlled methods of fluoride administration.

Milk has also been proposed as a viable fluoride vehicle since it is consummed most commonly by those in greatest need of fluoride exposure, ie. children and pregnant mothers as, in addition to the potential dental benefits, there are plentiful vitamins, calcium and phosphorous present. Stephen *et al*. (1981) have demonstrated that a caries reduction of 73 per cent in erupting first permanent molars is attainable but, overall, the 5 year

benefit of school-based distribution was 38 per cent. Besides, implementation would be expensive with difficult quality control from many outlets should such a policy be implemented on a large scale.

The most commonly used topical fluoride preparation is dentifrice. This provides an excellent vehicle and in the U.K., fluoridated dentifrice usage over the past 15 years has increased from 15 to 97 per cent of the total market share (Dowell & Joyston-Bechal, 1981). Toothpaste is employed as an adjunct in the maintenance of oral health through the mechanical cleansing of the oral tissues. Nevertheless, adults can greatly benefit from the use of topical fluorides (Russell & Elvove, 1951) and dentifrices would seem an ideal means of providing frequent fluoride exposure to enhance early enamel lesion remineralisation. It is important that the fluoride should not react with any of the other dentifrice constituents, von der Fehr & Moller (1978) having shown that when combined with either dicalcium phosphate or calcium carbonate, the anticaries activity of dentifrice fluoride was lost. As a result, in recent years the abrasive systems have been changed and the source of fluoride has come from either sodium fluoride or sodium monofluorophosphate. Most of the fluoride now remains in a soluble reactive phase in the new dentifrice formulations, although it is thought to lose some activity after 6 months. There will be further discussion on the place of fluoridated dentifrices in the remineralisation process in Chapter 5.

Perhaps the next most common means of fluoride delivery to children is by drops and tablets. Fluoride drops/tablets from birth have been cited in various studies as an alternative to water fluoridation. Marthaler (1969) reported a 47 per cent caries reduction over an 8 year period when tablets were given daily at school, by teachers. In contrast, Stephen & Campbell (1978) reported an 81.3 per cent caries reduction in first permanent molars when school children were asked to let 1 mgF tablets slowly dissolve in the saliva, thus enabling fluoride concentrations of around 1000 ppmF to be achieved. Since then, fluoride drops have appeared as a more suitable oral dosage form for youngsters. There is, as yet, a paucity of data in the literature pertaining solely to fluoride drops and later fluoride tablet supplementation studies.

Fluoride may also be combined with proprietary vitamin supplements (Aasenden & Peebles, 1978), although similarly, the dose has to be adjusted to account for environmental fluoride levels. In addition, fluoride exposure has to be continued long after the need for vitamin supplements has ended. Nonetheless, this method of fluoride implementation has been somewhat neglected as most mothers, expectant, nursing and _de facto_, appreciate the importance of vitamin supplements during pregnancy and childhood. Indeed, supplementation is often pursued by mothers for some years after the actual need has diminished. Hence, such formulations could well have a place as Hamberg (1971) has previously demonstrated their

50 per cent caries-inhibiting potential up to 6 years of age.

1.3.7 Discussion

Despite the continuing discussion about the exact mechanisms of fluoride action, there is little doubt that:

1. enamel formed, especially during its later stages, in a fluoridated environment will be more resistant to acid dissolution,
2. enamel which, prior to eruption, has been exposed to little or no fluoride, will still benefit from topical exposure even although enamel maturation is complete,

   \- and -

3. frequent exposure to fluoride is necessary to maintain a high resistance to caries attack especially during acid attack

   \- but -

4. much confusion remains with regard to the ideal vehicle, concentration, duration and frequency of exposure.

Thus, the purpose of the work reported in this thesis was to attempt to devise a model which would help rationalise some of the problems discussed above.

1.4 CARIES MODELS

1.4.1 Introduction

The use of natural white spot lesions as a source of experimental material for remineralisation studies suffers from two major disadvantages. Firstly, the history of the lesion is unknown, since it will have undergone both de- and remineralisation while exposed to the oral environment. Secondly, suitable natural lesions are difficult to obtain, even on teeth which are removed for orthodontic purposes. There was a need therefore, to have a laboratory technique for the controlled demineralisation of enamel. Several methods have been proposed and all have involved proton attack, either from natural plaque (Clarkston et al., 1984a; Geddes et al., 1986), acidified gels (von Bartheld, 1958, 1961; Gray & Francis, 1963; Silverstone, 1966, 1967; Groeneveld, 1974; Ingram & Silverstone, 1981), or liquid solutions (Francis & Briner, 1973; Featherstone et al., 1978; van Dijk et al., 1979; Weatherell et al., 1983).

This section will briefly discuss the choice of dental tissue used in this study, and outline the advantages and disadvantages of the artificial caries systems mentioned above.

1.4.2 Experimental dental material

A wide selection of dental enamel sources has been previously used in experimental caries research, including human, bovine, ovine and equine tissue. Featherstone & Mellberg (1981) reported that lesions progressed in permanent bovine and ovine enamel at nearly three times the rate in permanent human enamel. Human dental tissue is, therefore, the material of choice for human remineralisation studies, since extrapolating results from bovine or ovine tissue to the human clinical situation may prove erroneous.

1.4.3 Artificial lesion production by natural plaque

Recently, a technique for creating artificial carious lesions using natural human plaque has been proposed (Clarkston _et al_., 1984a; Geddes _et al_., 1986). This involved the application of human, one day old plaque to the surface of an enamel slab. The plaque was supplied with a carbohydrate substrate and incubated for 24 hours at 37°C. Lesion production occurred in 5 - 10 days, although the rate of mineral loss was difficult to control and lesions often failed to exhibit the characteristics of the natural carious lesion, eg. the retention of surface shine. The technique is tedious and it would seem unlikely it could be employed for the production of artificial lesions on a large scale. However, to its advantage the use of natural plaque would appear to hold much potential and it is hoped that future investigations,

especially with regard to the cariogenicity of foodstuffs, will provide useful information regarding the carious process.

1.4.4 Acidified gel techniques

In this technique, crowns of teeth previously covered with acid resistant nail varnish, apart from test exposure windows, are immersed in an acidified gel for periods up to 12 weeks. The acidified gel used in the production of artificial enamel lesions is made from gelatin (von Bartheld, 1958, 1961; Silverstone, 1966, 1967), hydroxyethylcellulose (Gray & Francis, 1963; Groeneveld, 1974) or methylcellulose (Ingram & Silverstone, 1981). The gel is normally combined with a source of calcium and phosphate, and acidified with lactic acid, to provide the appropriate proton attack. The ability of the gelatin to produce lesions has been attributed to its high content of impurities which "protect" the enamel surface (Featherstone *et al*., 1978) and its often unknown level of fluoride (Pearce, 1983; Borsboom *et al*., 1985). Indeed, Borsboom *et al*. (1985) have claimed that if no fluoride is present, then subsurface lesion formation will not occur, whereas Anderson & Elliott (1985) have stated that supersaturation of the demineralising solution, anatomical features or chemical gradients are not required for subsurface lesion formation. Also, Moreno & Zahradnik (1974) have shown that the presence of calcium and phosphate in a demineralising solution is fundamental to the creation of demineralisation without loss of

structural integrity, in agreement with the later work of Weatherell et al. (1983). In the earlier studies of von Bartheld (1961) and Silverstone (1966, 1967), no mention is made of the presence of calcium, phosphate or fluoride content of the demineralising gel. However, it can always be presumed that these elements must have been present as gel contaminants, according to Featherstone et al. (1978); Pearce (1983); and Borsboom et al. (1985).

Despite the long periods necessary for lesion production and unpredictable impurity content, the acidified gel usually produces artificial lesions which are both histologically and radiographically similar to the early carious lesion (Silverstone, 1973).

1.4.5 Buffered solution methods

The use of buffered solutions differs very little from acidified gels. The technique involves the use of a surface dissolution inhibitor, such as methane diphosphonate (MHDP) in a lactate buffered solution. Also employed has been a buffered solution alone, exhibiting a well defined degree of saturation with respect to hydroxyapatite (Francis & Briner, 1973; Featherstone et al., 1978; van Dijk et al., 1979). The successful creation of subsurface lesions using the latter technique has stressed the importance of calcium and phosphate in a demineralising solution. In contrast however, Featherstone et al. (1978) suggested that the MHDP alone, as a surface protector, was responsible for the successful

creation of the intact surface layer. It was claimed that the MHDP selectively displaced phosphate ions initially from the enamel surface before being reversibly adsorbed to the outer surface, protecting it against further direct dissolution. It was also claimed that impurities in any other system which successfully creates subsurface demineralisation must provide this surface protecting role. From the above literature review there would seem to be confusion as to the exact mechanism of the formation of the intact surface layer.

Those workers who employ buffered solutions claim the advantages of excellent control of pH and contaminants. However, there is little information given in the literature regarding the duration of exposure necessary to successfully create subsurface lesions. From the author's experience, the use of either a saturated or supersaturated solution demands that the solution be changed frequently due to crystal formation. Therefore, there is an increase in laboratory handling time in contrast to that required when using acidified gels.

1.4.6 Acid vapour technique

Weatherell et al. (1983) have proposed the technique of exposing enamel surfaces to a moist acid vapour. Here, teeth were suspended over a number of different acids for periods of between five and sixty-five hours. A droplet of vapour was seen to collect on the enamel surface, and in most cases there was the formation of a subsurface

lesion. An analysis of both the droplet and the lesion was then carried out.

Soon after the initial acid attack, crystal formation was noted within the acid vapour droplet, thus supersaturated conditions must have been present. The procedure, lacking in the use of plaque, acidified gels, buffers and weak acids, restricted confusing background chemical contamination to a minimum by establishing the simplest of conditions. The appearance of the histological zones within the carious lesion were explained against the background of supersaturation and crystal growth, with subsurface demineralisation being attributed to a net loss of mineral resulting from crystal growth outside the tooth. They proposed that initial carious lesions form as a result of low supersaturation with respect to calcium and phosphate at the enamel surface.

One of the most striking features of the above technique is that a subsurface lesion actually forms, as a more expected result might have been erosion of surface enamel similar to that obtained by etching. As stated previously (1.4.4), with regard to the acid gel technique, the presence of calcium and phosphate in a demineralising solution (the droplet in this case), would appear crucial if surface integrity is to be maintained. However, contaminants from the tooth, such as fluoride, may have leached into the droplet itself and contributed to its surface-protecting role as referred to by Featherstone _et al_. (1978) and Borsboom _et al_. (1985).

1.4.7 Discussion

In this project, the need for artificial enamel lesions which exhibited a demineralisation pattern similar to the natural carious lesion was considered important. Each of the techniques discussed previously have advantages and disadvantages. The acidified gel, despite its impurity content has, in the author's experience, consistently produced lesions which are histologically similar to natural caries. Thus, in spite of the relatively long exposure periods necessary for lesion production, the gel technique was considered the method of choice.

1.5 EXPERIMENTAL METHODS

1.5.1 Introduction

In this section previous _in vitro_, _in vivo_ and _in situ_ experiments will be discussed, and the advantages of experiments which are carried out within the oral environment will be outlined. In addition, a comparison will be made between the use of enamel slabs and sections, and the advantages and disadvantages of each will be discussed.

1.5.2 The comparison between enamel slabs and sections

Both enamel slabs and sections have been used in remineralisation studies. With slabs, only a single measurement of mineral content can be made at the end of an experiment and, in addition, a separate control slab is necessary. However, to investigate any of the parameters affecting de- or remineralisation, there is a need for sensitivity to small changes in mineral content (Smits & Arends, 1985; Strang et al., 1986). Unfortunately, enamel is not a homogeneous material and does not show a constant level of demineralisation when subjected to a constant acid attack (Groeneveld et al., 1975; Strang et al., 1986). Harvey et al. (1982), and Featherstone & Silverstone (1982) attempted to overcome the problem of enamel variation by using enamel sections in vitro, the mineral content of which could be measured at any point during the experimental period and related to the baseline value. This technique involves the production of sections at the start of the experiment, in contrast to previous procedures in which sections were cut at the end of the test. The major advantage of such a procedure, is that provided the specimens are thin enough, then examination of the section can be carried out at any point during the procedure. Strang et al. (1986) have previously reported that there is greatest variation in lesion mineral content in sections taken from different teeth, and least in repeat measurements of the same lesion. The limit of sensitivity to measure changes in mineral content in the single section technique is the repeatability of the

measurement of the mineral content. Whereas, with the enamel slabs the variation in the mineral content between slabs is dominant (Strang *et al.*, 1986). The variation in lesion mineral content in sections taken from different teeth and even from the same tooth are much greater than that of repeat measurements of the same lesion. This topic will be discussed in greater detail in Chapter 3.

By alleviating the need for separate control tissue, the use of single sections would seem to be the technique of choice where there is a need to record small changes in mineral content.

1.5.3 *In vitro* techniques

There has been an extensive number of laboratory investigations carried out to study both de- and remineralisation. *In vitro* techniques to create demineralisation have been discussed in some detail previously (1.4). This section will be concerned only with *in vitro* remineralisation investigative methods, briefly outlining the major advantages and disadvantages of each.

Laboratory remineralisation methods have ranged from the simple, where entire teeth or enamel sections were left in dentifrice slurries for a single exposure of up to 40 hours (Joyston-Bechal & Kidd, 1985), to the more complex solution cycling of ten Cate & Duijsters (1982), or the elaborate constant composition technique of Buskes *et al.*

(1985). All have attempted to quantify the effect of fluoride either on remineralisation, or the prevention of further mineral loss. From the literature, the influence of fluoride on de- and remineralisation has been investigated from a variety of different angles. For example, it has been well established that levels of fluoride ranging from 0.12 to 5.0 ppm are sufficient to either influence the pattern of mineral loss, or inhibit demineralisation entirely (Koulourides et al., 1961; Levine, 1975; ten Cate & Duijsters, 1983a; Borsboom et al., 1985). Ten Cate & Duijsters (1983a) concluded that a fluoride concentration which is saturated with respect to the formation of calcium fluoride, rather than fluorapatite, is of importance.

There is general agreement that the formation of calcium fluoride is an important intermediate product of remineralisation and may act, because it is slightly soluble, as a fluoride reservoir from which it can be released both into the lesion and the external environment (Leach, 1959; Mellberg et al., 1966; Kirkgaard, 1977 a,b; Fejerskov et al., 1981; Dijkman et al., 1986). Kirkgaard (1977 a,b) concluded there was little correlation between dentifrice fluoride concentration and the fluoride concentration in the surface layer. Indeed, Joyston-Bechal & Kidd (1982) have claimed an "optimal" level of fluoride, the value of which was not specified. With regard to frequency and duration of exposure, results are often conflicting. For example, Joyston-Bechal & Kidd (1982) claimed that more fluoride is deposited with

frequent short exposures, whereas Arends & Schuthof (1981), and Retief *et al*. (1980, 1983) maintained that a 24 hour application of fluoride resulted in greater uptake than with a 3 min application. While laboratory techniques, in most cases, do not attempt to mimic the natural oral environment, they have the advantage of being able to accurately control any number of parameters at one time. This cannot be duplicated *in vivo*, due to the large number of uncontrolled and variable parameters present within the oral ecosystem.

In conclusion, laboratory investigations into the role of fluoride have been extensive but inconclusive as to the rational use of topical fluorides in successful caries prevention.

1.5.4 *In vivo* and *in situ* techniques

Studies performed within the natural ecosystem of the oral environment have inherent advantages over the *in vitro* situation. These were pioneered by von der Fehr (1966), who used gold clasps in close proximity to the buccal surfaces of teeth destined for extraction. An artificial stagnation area was created, and white spot lesions developed as a result of the plaque build up. Later, von der Fehr *et al*. (1970) carried out a similar study where subjects were asked to refrain from all oral hygiene and to supplement their diets with sucrose rinsing nine times per day. At the end of a 23 day period, there was distinct evidence of white spot lesions on the labial

aspects of most teeth of each dentition. On the whole these lesions were reversible when rigorous oral hygiene and topical fluoride therapy was re-established. The procedure was later repeated by Edgar _et al_. (1978), although only for 8 - 10 days, when lesion appearance was again noted. However, there is doubt that such a protocol would receive ethical approval today.

Attempts have been made to overcome these difficulties by Holmen _et al_. (1985 a,b), who employed orthodontic bands as a means of creating stagnation areas. These bands were placed on individual teeth destined to be extracted for orthodontic purposes. In those studies, a gap was created between the band and tooth, in which plaque was allowed to accumulate and demineralise the underlying enamel. Four teeth were then removed from the same individual, one each after 1,2,3 and 4 weeks' cariogenic challenge, to assess the progressive stages of the carious process in enamel. Orthodontic bands have also been employed for the monitoring of bacterial colonisation and metabolism (Arneberg _et al_., 1984; Minah _et al_., 1984). In general, the technique lacks sensitivity since a separate control specimen is necessary.

An _in situ_ technique has been proposed, which has avoided many of the ethical problems of the _in vivo_ techniques discussed above. This involves the mounting of enamel slabs in dental prostheses (Koulourides _et al_., 1974), and has been employed for a number of studies. These have included the quantitative assessment of lesion rehardening

(Koulourides *et al.*, 1974; Gelhard *et al.*, 1979; Featherstone *et al.*, 1982; Smits & Arends, 1985; Essig *et al.*, 1985), the measurement of fluoride uptake by porous enamel (Mellberg & Chomicki, 1983; de Bruyn *et al.*, 1985 a,b; Stookey *et al.*, 1985), and the investigation of lesion permeability (Brudevold *et al.*, 1984).

The technique has contributed a number of valid observations regarding the dynamics of the carious process and remineralisation. Koulourides *et al.* (1974) showed that fluoride had an affinity for porous enamel while Mellberg & Chomicki (1983, 1985a,b), and Smits & Arends (1985) demonstrated that the incorporation of fluoride during remineralisation encourages deposition of a more stable mineral than if fluoride had been absent. Featherstone *et al.* (1982) observed variation from person to person by demonstrating that some individuals appear to remineralise better than others, and that in some individuals saliva alone would appear to be a potent remineralising solution.

The use of slabs, however, has inherent disadvantages due to the mineral and caries susceptibility variations which occur between teeth, and even within the same tooth (Groeneveld *et al.*, 1975; Strang *et al.*, 1986). Since only two slabs can be embedded in one prosthesis at a time, a relatively large number of subjects and specimens is required for any study to which statistical analysis can be applied. Furthermore, Smits & Arends (1985)

concluded that variation between enamel blocks may obscure any possible remineralisation effects of any test solution. Thus, the use of slabs may not be sensitive enough to investigate small differences in fluoride concentration and/or frequency of exposure.

1.6 METHODS OF ASSESSMENT

1.6.1 Introduction

A number of methods have been employed to assess the changes in mineral content occurring from normal through to carious enamel and, in turn, the mineral redeposition during remineralisation. This section will discuss the various techniques.

1.6.2 Microradiography/microdensitometry

In this technique thin sections of enamel are mounted on high resolution radiographic plates and exposed to a monochromatic X-ray source. The radiographic density of the enamel lesion can then be scanned, calibrated against the density of known aluminium thicknesses, and readily computerised to permit calculation into volume per cent mineral using the equation of Angmar _et al_. (1963). Microradiography has been described as an ideal method for the quantification of normal enamel (Soni & Brudevold, 1959a, Angmar _et al_., 1963) and assessment of mineral movement during de- and remineralisation (Angmar _et al_., 1963; Bergman & Lind, 1966). It provides detailed

information on the degree of mineralisation at the microscopic level, and is unaffected by the presence of water or mineral form. Used initially by Bergman & Lind (1966) for the investigation of demineralised tissue, it has been employed extensively to monitor mineral deposition within porous enamel (Theuns et al., 1980; ten Cate & Duijsters, 1983b; Pearce, 1983). Although not differentiating between varying forms of mineral within the enamel lesion, microradiography is a simple, accurate and reproducible technique (Featherstone et al., 1983) and will be described in more detail in Chapter 2.

1.6.3 Polarised light microscopy

Polarised light has been used extensively as a semi-quantitative and qualitative method of carious enamel assessment. The mineral component of enamel exhibits birefringence since it can resolve a beam of plane polarised light into two rays which travel at different velocities. The enamel crystals themselves have an inherent birefringence of known value (1.62) called "intrinsic birefringence". Enamel, therefore, has two refractive indices, related to the two planes of transmission within the crystal. The use of quantitative polarised light microscopy in caries research attempts to relate the difference between the two planes of light, to differences in pore volume.

Apart from the mineral and organic constituents of enamel, there are minute spaces present. During carious

dissolution, there is an increase in the total volume of the "spaces". These spaces can be filled with media of known refractive index and a measure of the volume or percentage of the spaces carried out. This results in a second type of birefringence called "form birefringence", which may be altered by changing the imbibing media. Using this technique, Silverstone (1966, 1967, 1968) and Kidd (1983) have described the histological pattern of the carious enamel lesion in some detail, where four distinct zones have been reported. These are the surface, dark and translucent zones, and the lesion body. The appearance of a dark zone within the lesion is thought to be indicative of either a slowly progressing lesion, or further deposition of mineral within the lesion ie. remineralisation. The micropore system of the dark zone has been described as a molecular sieve (Darling et al., 1961; Poole et al., 1963) and is considered the "resting point" for mineral which has been removed from the deeper layers of the enamel lesion, and which may be transported to more superficial parts of the lesion at a later stage.

Remineralisation studies using polarised light attempt to quantify alteration in porosity during mineral deposition, by measuring changes occurring in both the surface zone and the dark zone. As mineral is deposited, both zones are thought to become broader and more well-defined (Kidd, 1983).

Polarised light, as the sole routine quantitative method of lesion assessment is limited, since it is both time-

consuming and has recently been shown to underestimate lesion porosity (Shellis & Poole, 1985). Previous pore volume calculations assumed even distribution of the pores, but Shellis & Poole (1985) showed this is not so, and that pore distribution within the lesion is more random than originally assumed. It was concluded that polarised light must be used in conjunction with other quantitative techniques for accurate pore-volume assessment.

1.6.4 Microhardness

Enamel microhardness measurements involve indenting the enamel surface with a Knoop diamond under the action of a known weight. By measuring the dimensions of the indentation, an assessment of the lesion's resistance to breakdown can be carried out and has been used extensively on enamel slabs which were exposed to the oral environment (Koulourides et al., 1974; Gelhard et al., 1979) and in vitro (Purdell-Lewis et al., 1976; Arends et al., 1979, 1980). This technique has been employed on the polished cut surface of the enamel lesion as well as on the anatomical surface. Various claims have been made as to which is the better measure of lesion de- or remineralisation. It was concluded by Arends et al. (1980) that the results of natural enamel surface indentation were in good general agreement with microradiographic and scanning electron microscope assessment (vide infra). In contrast, ten Cate et al. (1985) claimed that microhardness should always be carried

out on the polished cut surface, as this differentiated between varying mineral deposition within the lesion, which indenting the anatomical surface does not. Microhardness does not measure mineral content directly, but assumes a constant relationship between lesion resistance and mineral content. The technique is however, both time-consuming, operator dependent and destructive (Arends et al., 1980).

1.6.5 Chemical analyses

The chemical analysis of enamel biopsies taken by applying an acid etchant to the enamel has normally involved an assessment of lesion fluoride profile (Aasenden et al., 1972; Retief et al., 1980; Bruun et al., 1983; Pearce, 1983; Retief et al., 1983; Chow et al., 1985). Hallsworth et al. (1972) have proposed an elaborate system of lesion microdisection, and later chemical analyses of lesion mineral and trace element content. Other analyses have included the assessment of iodine permeability (Brudevold et al., 1984) and the elaborate technique of Ingram & Silverstone (1981) to measure total mineral loss from enamel during early lesion formation. In general, there has been good agreement between chemical analyses and other methods of lesion assessment (Ingram & Silverstone, 1981; ten Cate & Duijsters, 1983a). The techniques are again both time- consuming and destructive.

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1.6.6 Scanning electron microscopy / electronprobe

The scanning electron microscope (SEM) has been employed alone, or combined with the electronprobe, to study both the natural and fractured enamel surfaces of the early enamel lesion (Haikel et al., 1983; Fejerskov et al., 1984; Holmen et al., 1985 b,c). The SEM has the advantage over techniques using light microscopy in that it is not restricted by the resolution of visible light and fine detail can be examined at high magnification. It has been employed to examine differences in surface morphology (Ingram & Fejerskov, 1986) and the interprismatic region of fractured enamel (Brannstrom et al., 1980). Ingram & Fejerskov (1986) concluded there were no measurable differences in surface morphology between normal and carious enamel. The technique is restrictive as a quantitative method and is destructive because the specimen has to be coated before examination within the SEM vacuum.

The SEM has been used in conjunction with the electron probe in a number of studies to assess lesion progress (Purdell-lewis et al., 1976; Clarkson et al., 1981; Clarkson et al., 1984b). In general, there has been good agreement between the different methods of quantification but, like microhardness, the electronprobe technique is destructive.

1.7 DISCUSSION

There is clearly a need for extensive well-controlled remineralisation studies, preferably within the natural environment of the oral cavity, to assess the true effects of fluoride concentration, frequency and duration of exposure. The oral environment is difficult to emulate in the laboratory, therefore, an in situ investigative technique where samples can be removed for assessment, would seem to have considerable potential. However, to date, in situ techniques have involved the use of slabs, the mineral content of which can only be measured accurately at the end of an experiment. The use of sections, in which repeat measurements could be made at various times during an experimental procedure and later related to the baseline value, would increase the sensitivity for detecting small changes in mineral content. If the inherent advantages of the single section technique are to be successfully extended to the in situ situation, then there is a need for a quantitative, repeatable and simple method of assessing mineral content.

From the above review, quantitative microradiography/ microdensitometry appears as the only technique which enables the accurate measurement of mineral content deposited within the enamel lesion. Coupled with the use of single enamel sections, the result should provide a model which would appear sensitive enough to investigate small changes in many of the parameters affecting both de- and remineralisation. This was the method chosen for use

in this study and a more detailed description of the microradiographic technique will, therefore, be outlined in section 2.6.

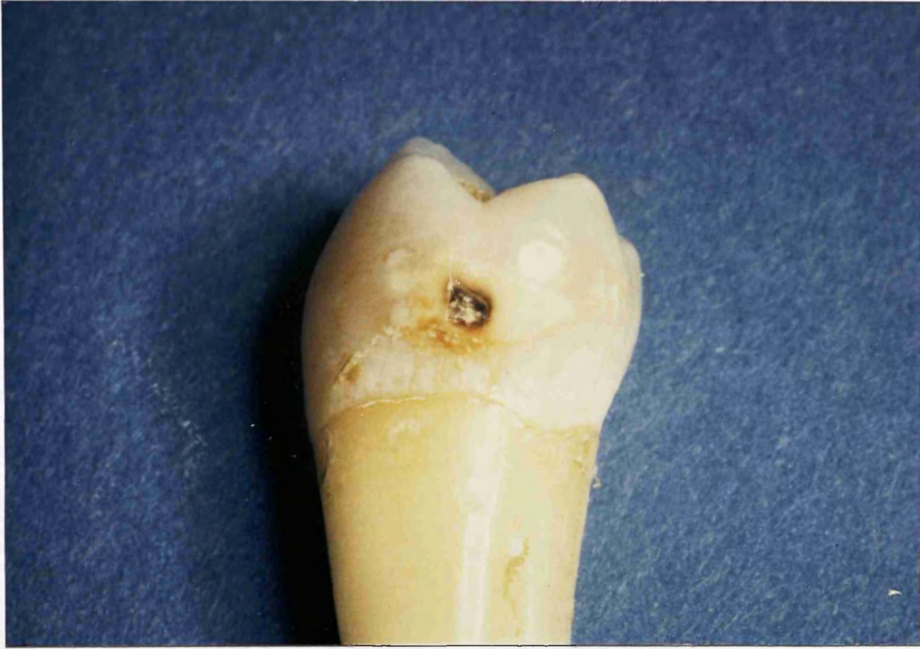

Figure 1.1 A 'white spot' lesion on the distal aspect of an extracted premolar, illustrating early breakdown of the tissue, resulting in cavitation.

TABLE 1.1 RANGE OF CONCENTRATION, FREQUENCY AND MODALITY INVESTIGATED IN PREVIOUS FLUORIDE STUDIES

PARAMETER	RANGE
Vehicle	drops, tablets, rinses, milk, dentifrices, gels and varnishes
Concentration	1 - 29,000 ppmF
Frequency	from twice/day to once/year

CHAPTER 2 METHODS & MATERIALS - GENERAL

2.1 INTRODUCTION

In this chapter, the methods used to collect and prepare the teeth employed in the experiments, the production of artificial carious lesions, the sectioning and grinding of the specimens and the techniques used to quantify the mineral content of the lesions will be described. Most of these techniques were standard ones, but others have been developed and modified by the author for the purposes of this project.

2.2 TOOTH SELECTION AND PREPARATION

2.2.1 Source, preparation and examination of the teeth

Human premolar teeth were employed, since it has been shown that artificial lesions formed in enamel from other sources such as bovine and ovine samples, progress at up to three times the speed (Featherstone & Mellberg, 1981). (See also section 1.4.2).

The teeth were obtained from dental practices and oral surgery units in and around the Glasgow area (water fluoride < 0.03 ppm) and were stored in a 5 % aqueous thymol solution until required. The enamel surface was cleaned with a rubber cup and a fine pumice-alcohol mixture to remove any pellicle or calculus deposits

thereon. The crowns were thoroughly cleaned in alcohol, and then in warm soapy water. After drying in warm air, the entire crown was examined under x 10 magnification to check for flaws, cracks or incipient carious lesions on the buccal enamel surface.

2.2.2 Varnishing and window preparation

The crowns of the teeth were entirely covered in a coat of a proprietary nail varnish (Max Factor, London, England), as illustrated in Fig. 2.1. After 1 - 2 hours, the buccal aspects of the teeth were given a second coat, to ensure adequate thickness of varnish. The coated teeth were stored for at least 24 hours, to allow the varnish to set. Using x 10 magnification, four to ten exposure windows, (dimensions approximately 1 x 5 mm), were cut on the buccal surface of each tooth with a No. 11 scalpel blade (Swann-Morton Ltd., Sheffield, England). One such tooth is shown in Fig. 2.1. To ensure that the underlying enamel had not been damaged, the varnish was removed from a representative sample by acetone application and the teeth were examined carefully for surface scratches. At no time, however, was damage noted on any of the teeth examined, and the prepared samples were then ready for immersion into the artificial caries system (<u>vide infra</u>).

2.3. THE LACTIC ACID GEL ARTIFICIAL CARIES SYSTEM

2.3.1 Chemical details

The chemical methods used to produce caries-like lesions in enamel have been described previously (1.4). The method chosen in this project was the acidified gelatin system (von Bartheld, 1958, 1961; Silverstone, 1967). Despite the long exposure times necessary for lesion production, the method consistently produces enamel demineralisation which is both histologically and microradiographically similar to the early incipient enamel lesion. The constituents and method of preparation of the acidulated gelatin used in this project are detailed in Appendix I.

Gelatin is a hydrophylic colloid obtained by the partial hydrolysis of collagen. It is composed of amino acids linked by peptide bonds to form linear polymers, and dissolves slowly in warm water forming a macromolecular colloid dispersion. The gelatin used (Difco Laboratories, East Molesey, Surrey, England) was employed for the creation of artificial lesions throughout the study. The gelatin was acidified and provided with a source of calcium and phosphate ions, their role in the external environment of the enamel being to regulate the mineral movement from gel to enamel. In addition, the presence of calcium and phosphate are considered fundamental to the successful artificial creation of an intact surface layer (Moreno & Zahradnik, 1974; Featherstone _et al._, 1978;

Weatherell *et al.*, 1983; Anderson & Elliott, 1985). The fluoride content of the gel was measured using an Orion Ionalyser Microprocessor (Orion Research, Massachusetts, U.S.A.) and found to be 0.1 ppmF.

2.3.2 Laboratory procedure

The production of 1 litre of the acidified gelatin is detailed in Appendix I. The pH of the gelatin was normally 4.0 for lesion production and a range of pH 4.0 - 5.0 for the further acid attack pilot study - see Chapter 7. Some difficulty was experienced in attaining a stable pH; thus, the gelatin was left for approximately 1 hour at a constant temperature of 37°C to ensure the pH measurement had stabilised.

The root of the tooth to be immersed was embedded in dental carding wax which was pressed firmly on to the inner surface of the plastic lid of a glass vial, and gently lowered into the bottle of molten gelatin, ensuring adequate thickness of gelatin overlying the exposure window region of the specimen. The vials were kept at room temperature for 10 - 12 weeks.

2.4 SECTION PREPARATION

2.4.1 Preparation of the enamel specimen

At the end of the acid exposure, the tooth was withdrawn from the gelatin, the varnish removed with amyl acetate (BDH Chemicals Ltd., Poole, Dorset, England) and the crowns washed thoroughly in warm soapy water. The buccal surface was examined using x 10 magnification to ensure the artificial caries process had not adversely affected the enamel surface, which should have retained both its structural integrity and shine. In addition, anatomical features such as the perikymata should still have been obvious. Most of the root was then removed using a dental drill (Milbro, Epson, England) and diamond disc, leaving approximately 1 - 2 mm of root dentine just apical to the enamel cap. The crown was subsequently halved mesio-distally, leaving the portion with the lesions on one cusp. The specimen was then mounted on to specially produced acrylic blocks using "Loc-tite" adhesive (Loctite (U.K.) Ltd., Welwyn Garden City, England). The block was left in a warm, dry, area for 1 - 2 hours to ensure adequate hardening of the adhesive.

Using a saw microtome (E. Leitz (Instruments) Ltd., Luton, England), sections were cut to an approximate thickness of 250 μm at the slowest approach speed setting on the microtome (Fig. 2.2). The slow approach speed procedure permitted the maximum number of sections to be cut from the brittle enamel block. Each section was

coded, using a graphite pencil, with a letter illustrating the experiment to which it would be allocated, and two numbers corresponding to (a) the tooth and (b) the section from which the specimen had been cut. By so doing, the section could be easily identified at a later stage, the code being retained for use once radiographic and microdensitometric analysis had been completed (*vide infra*).

2.4.2 A method of section grinding

Previous techniques for the production of thin enamel sections (Fearnhead, 1960; Fremlin *et al*., 1961; Bovis, 1968) were both laborious and time consuming. This section describes a technique which was both original and simple. Moreover, the method greatly reduced the time necessary for hand grinding of sections to the required thickness.

A heavy brass plate was covered in a damp gauze, as illustrated in Fig. 2.3. A slurry of carborundum powder (particle size - 0.3 μm; - Raymond A. Lamb, London, England) and water was mixed on a ground glass plate, previously used to sharpen soft tissue microtomes (Shandon Southern Products, Runcorn, Cheshire, England). The specimens, labelled on both cut aspects, were placed on to the moistened gauze, and slowly rotated round the glass plate in a "figure of eight" motion. The friction between the section and the cloth was greater than that between the section and the carborundum slurry on the glass plate,

thus the sections remained immobile on the gauze-covered brass plate. One of the cut surfaces was ground for only 10 - 20 sec, merely to remove the concentric rings caused by the saw microtome. The code was rewritten on that surface and the section ground until the required thickness was attained. Normally 4 - 6 sections were ground at any one time, with interuptions only to assess section thickness.

2.4.3 Measurement of section thickness

Section thickness was measured using a digital micrometer (Mitutoyo, Tokyo, Japan) as illustrated in Fig. 2.4. The micrometer had a flat table and a perpendicular measuring probe, which could be raised or lowered using a camera extension cable (Fig. 2.4). Measurements of section thickness were made at 1 mm intervals along the enamel face. Thus, this procedure also provided information regarding the planoparallelity of the section. Normally, six to ten measurements were made, the variation from cervical to coronal aspects rarely exceeding 1 - 2 μm. Section thickness within the range 100 - 120 μm was considered necessary to obtain an adequate microradiographic grey level range (vide infra).

2.5 MICRORADIOGRAPHY AND MICRODENSITOMETRY

2.5.1 Introduction

Microradiography and microdensitometry techniques for the analysis of dental hard tissues are based on the fact that the absorption of X-rays by an enamel section will depend on the mineral content of that tissue. Angmar et al. (1963) developed the theory for calculating enamel mineral content from microdensitometric measurements of microradiographs of enamel. Bergman & Lind (1966) extended the technique to assess the mineral content of altered, demineralised enamel. The application of the method has been employed extensively to assess the mineral content of normal enamel (Soni & Brudevold, 1959a) and the progression of mineral loss from enamel during demineralisation (Soni & Brudevold, 1959b, 1965; Groeneveld et al., 1975; Pearce, 1983; Feathersone et al., 1983; ten Cate & Duijsters, 1983b). However, limited work has been carried out using the technique for the investigation of remineralisation.

2.5.2 Theory

In general, quantitative microradiographic analysis of the carious lesion is based on the fact that demineralised enamel will absorb fewer X-rays than sound enamel. As stated above, the optical density (greyness) of a radiographic plate is dependent on the mineral content of the enamel. Thus, the lesion will appear as a dark area

on the radiographic plate, as shown in Fig. 2.5. Microdensitometric measurements enable the grey level to be quantified into mineral content for subsequent analysis.

Following the derivation of Angmar et al. (1963), the enamel is assumed to consist of two major components:

(i) an inorganic component of thickness "t_m", and

(ii) an organic element of thickness "t_o".

Thus, the grey level for any point in the lesion has resulted from X-ray absorption by both components. Hence, for a particular level of absorption of X-rays (ie. the grey value of a point on the lesion), the absorption in enamel can be equated against the absorption in an aluminium step wedge. Appendix II gives a detailed account of the formula derived by Angmar et al. (1963) equating the percent volume mineral with measured optical density of aluminium.

The following assumptions were made by Angmar et al. (1963):

(i) mineral salts have a density of 3.15

(ii) normal enamel has an average composition of 37.1 % Ca, 18.1 % P, 43.3 % O, 0.7 % C and 0.3 % H; which results in a Ca:P ratio of 2.05.

In addition, the assumption was made in this project, as in other remineralisation studies, that the Ca:P ratio in remineralised enamel was similar to that of normal and

demineralised enamel.

2.5.3 Microradiographic methods

Enamel sections of known thickness were mounted along with an aluminium stepwedge, on Kodak high resolution plates (Type 1A) (Eastman Kodak Company, Rochester, New York, USA) then placed in light tight holders (Fig. 2.6) and exposed to CuKα radiation from a Marconi X-ray tube (TX12) in an Enraf Nonius generator for 20 min at 20 kV and 30 mA. A diagramatic representation of the set-up is shown in Fig. 2.7. Plates were developed using standard techniques.

Initial studies indicated that the X-ray beam was not homogeneous, and exhibited a variation from "north" to "south". However, horizontally, beam variation was found to be less than 1 %. For this reason, the aluminium step wedge (thickness ranged from 50 μm - 300 μm) was placed along the Y-axis of the plate. This permitted microdensitometric measurements of the aluminium wedge to be made at the same level as the measurements of the lesion, thus benefiting from the X-axis beam homogeneity.

Radiation exposure (20 kV, 30 mA for 20 min at a target - specimen distance of 300 mm) and section thickness were chosen to optimise the grey level range for subsequent microdensitometric analysis.

2.5.4 Microdensitometric methods

Microdensitometry enables the grey levels in the radiographic plate to be quantified. The plate is illuminated by a stable light source and the light transmitted is detected by a sensor (Fig. 2.8). The sensor produces an electrical signal proportional to the transmitted light, and hence the signal is related to the greyness of the point beneath the radiographic plate.

Two microdensitometers were used in this study:

(i) Joyce-Loebl - Type 3CS

-and-

(ii) Leitz ASBA image analyser

Most of the analyses in Chapter 3 were carried out using the Joyce-Loebl, whereas all the analyses in chapters 5 and 6 were performed using the ASBA image analyser. Unfortunately, the latter microdensitometer did not become available until the final part of the project.

The Joyce-Loebl (Fig. 2.9) uses a photomultiplier to detect transmitted light. In this instrument a narrow light beam (100 μm x 10 μm) is scanned over the radiographic plate. The output from the photomultiplier is amplified and displayed on a chart recorder. A typical analogue output tracing (optical density -vs- distance) of an enamel lesion and concomitant aluminium stepwedge is illustrated in Fig. 2.10. This figure clearly shows the surface, the minimum and the maximum optical density levels of the lesion and normal enamel. The analogue

tracings were digitised into a Plessey microcomputer (Plessey Peripheral Systems, Surbiton, Surrey, England), calculation of mineral content of each point carried out, and the data stored for further analysis.

The Leitz Image Analyser is shown in Fig. 2.11. The major difference between this unit and the Joyce-Loebl is that the whole lesion is illuminated and the light transmitted is detected using a video camera. It consists of a microscope (Leitz Dialux 22) with a stabilised power supply, and a video camera (ASACA Corporation Type 700BE) mounted on the microscope. The analyser unit, controlled by a Z8002 microprocessor, allows the video signals from the camera to be digitised into 256 grey levels with a resolution of 256 x 256 pixels. Potentiometers on the ASBA front panel, enable the unit to be set up so that the 256 grey levels cover the region of interest. The digitised image is then transferred to a BBC-B computer for further analysis and storage of results. The software for both the ASBA and the BBC-B computers was written by Dr. R. Strang and Mr. I.P.A. Macdonald (Department of Clinical Physics and Bioengineering, West of Scotland Health Boards).

Initially the radiograph was positioned on the microscope stage so that the thickest aluminium wedge was in the field of view. The intensity of the microscope light was adjusted to give maximum illumination without saturating the video camera. An area, 64 x 64 pixels (192 x 192 μm), was sampled in the centre of the screen and the average

grey level corresponding to that wedge calculated and transferred to the BBC computer. The grey levels for the other thicknesses of the stepwedge were calculated and transferred in turn. The radiograph was then positioned so that the enamel lesion was in the field of view and the camera rotated until the enamel surface was horizontal on the ASBA monitor. The image was then digitised into 256 x 256 pixels and transferred to the BBC computer. At the standard magnification used, one pixel corresponds to 3 μm. Software, written for the BBC computer, colour coded the grey levels in the image and redisplayed the lesion on the BBC computer monitor (Fig 2.12). At this point, using the keyboard, the width and position of the area of interest on the lesion could be altered for further analysis. This was shown by vertical lines on the monitor (Fig 2.12). The computer then calculated the average microdensitometric profile within these lines and displayed it at the side of the screen (Fig 2.12). At this stage, the profile was still in terms of grey level. Horizontal lines were then positioned over the image to limit the profile information stored by the computer. For baseline measurements of mineral content of a particular lesion, a hardcopy of the screen (i.e. Fig 2.12) would be obtained on the printer. This enabled the area of interest in subsequent radiographs of the same lesion to be positioned accurately to within 1 pixel.

A fourth-order polynomial was then fitted to the aluminium wedge grey levels and the grey levels of the profile converted to per cent volume mineral using the equation

derived by Angmar et al. (1963), as previously described. The profile and other relevant data were stored on floppy disc for subsequent analysis.

One of the major advantages of the ASBA unit was its ease of use. The apparatus also handled and recalled the data with great speed. In addition, repositioning of the lesion on subsequent plates, as described above, was carried out with accuracy. This latter feature, in particular, was not readily possible when employing the Joyce-Loebl. The other great advantage was the ASBA's speed of computation, one radiographic analysis taking approximately 5 min, compared to almost 40 min with the Joyce-Loebl.

2.5.5 Measured parameters

All lesion tracings were normalised to a sound enamel mineral content of 80 % prior to data handling. This procedure reduces the influence of second order effects on the calculated data, such as accidental marks on the radiographic plate. From the literature, the measured value for the normal enamel has varied from 87 % to 78 % (Groeneveld, 1974). The chosen value was considered well within the acceptable range.

In addition to the measurements of per cent volume mineral content of the surface zone and lesion body, the integrated mineral loss (IML) was calculated from the shaded area above the profile (Fig 2.13). The limits of

IML were taken from the 20 % level on the initial slope of the profile to a point (S) in sound enamel. This latter point was selected by the operator on the baseline profile of a lesion. In subsequent profiles of the same lesion, the point was calculated by the computer software to maintain a fixed distance between this point and the 20 % level point on the initial slope. In Fig. 2.13 the shaded area has units of percentage volume mineral x microns. However, IML is calculated in terms of fractional mineral content x depth, and hence has units of microns (μm).

Appendix II outlines in detail the derivation of the formula used to calculate the volume percent mineral from the optical density of the radiographic plate, which takes into account the absorption coefficients and thicknesses of the organic and inorganic elements of the enamel. This is equated against the absorption coefficient of the aluminium.

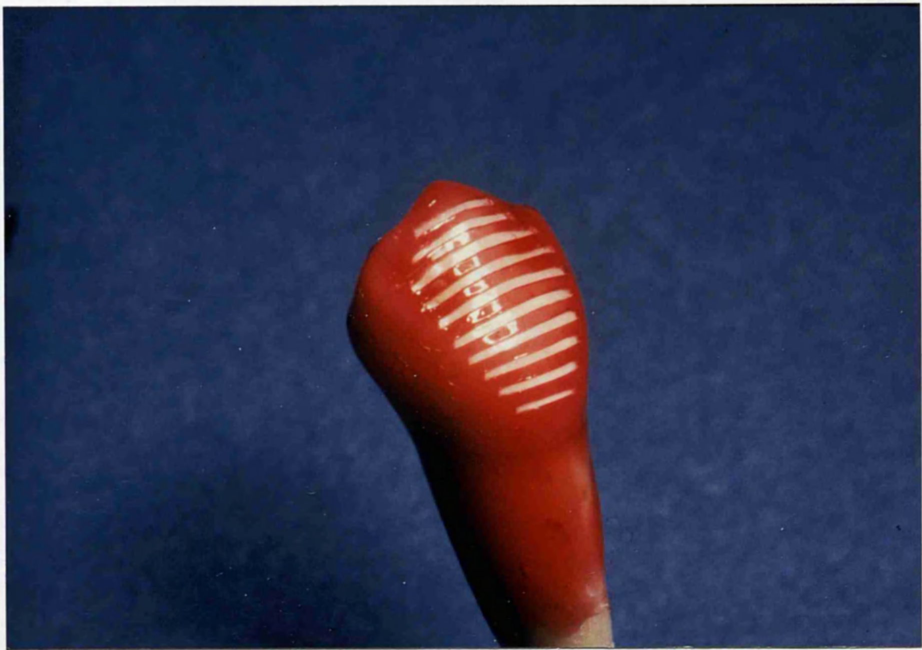

Figure 2.1 Varnished premolar tooth for immersion into the artificial caries system. Ten exposure windows have been prepared on the buccal aspect of the specimen.

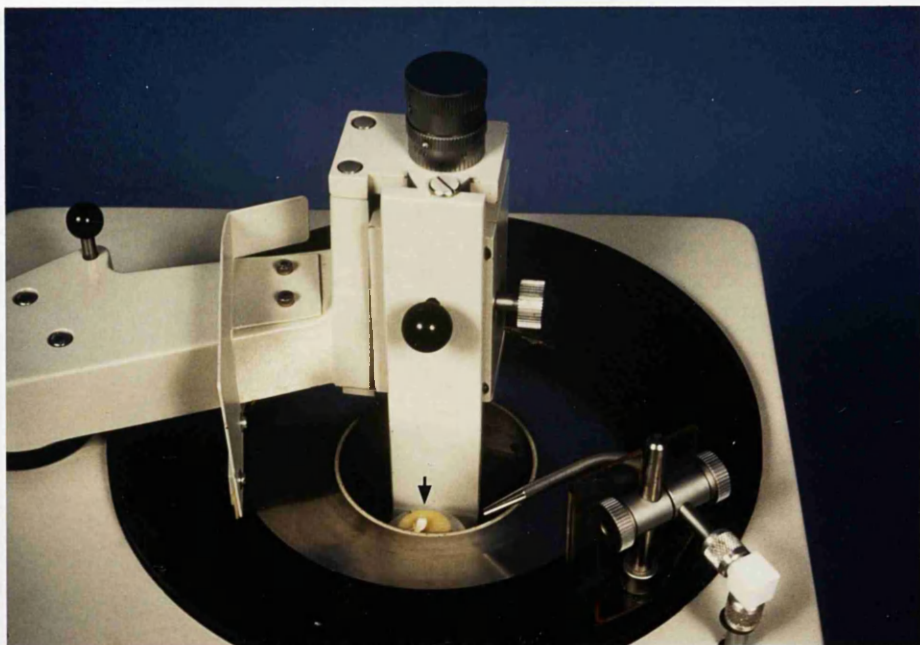

Figure 2.2 The Leitz saw microtome with a specimen mounted on the chuck (arrow). The diamond cutting aspect of the blade is water-cooled.

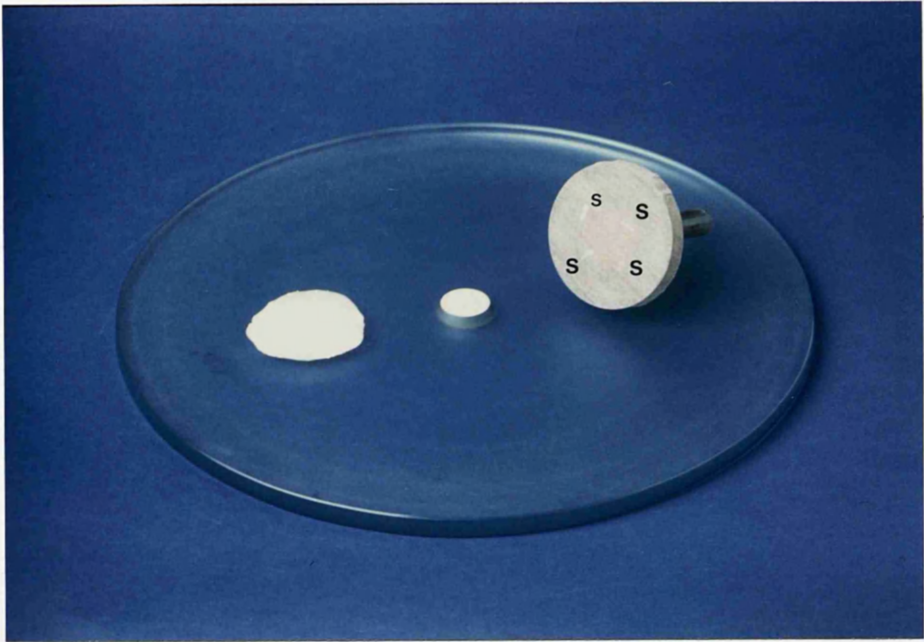

Figure 2.3 The ground glass plate with the carborundum powder slurry and the gauze covered brass plate. Four enamel specimens(S) can be seen adhering to the dampened cloth.

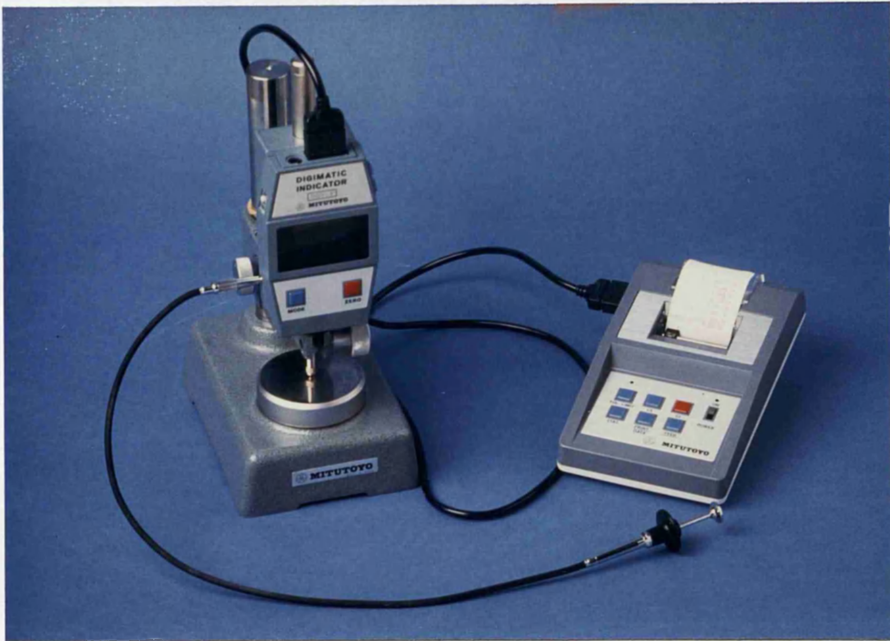

Figure 2.4 The digital micrometer employed to assess section thickness. The specimen is situated below the probe, lying on the flat table. The probe is lowered until contact is made and a readout of the measurement is produced on the printer.

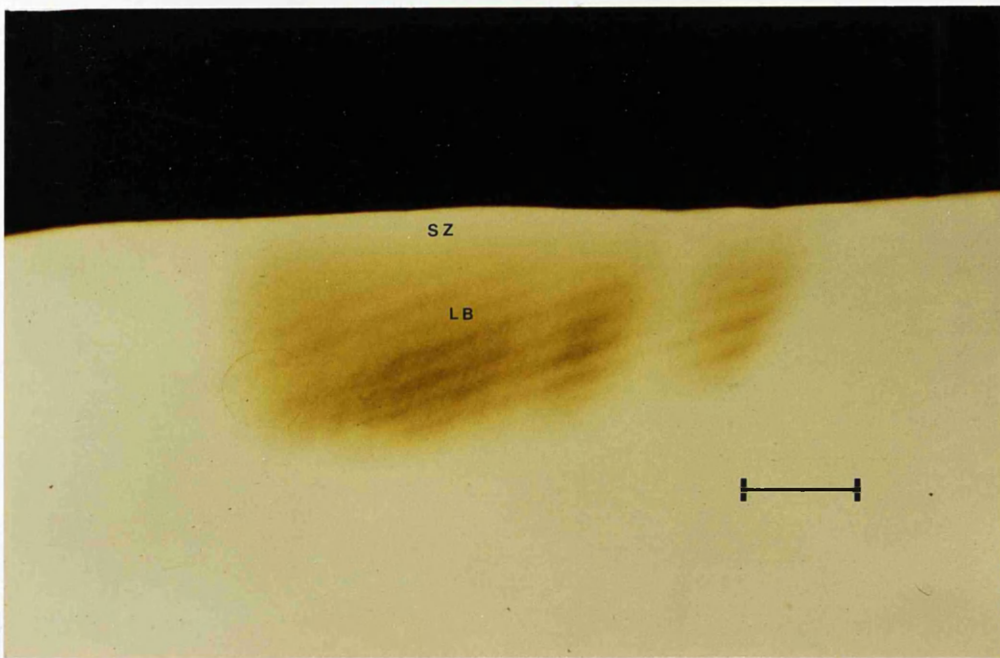

Figure 2.5 Typical microradiograph of an artificial enamel lesion. The normal enamel is white, in contrast to the dark area of the lesion. The surface zone (SZ) and lesion body (LB) are clearly visible. Bar is 100 µm.

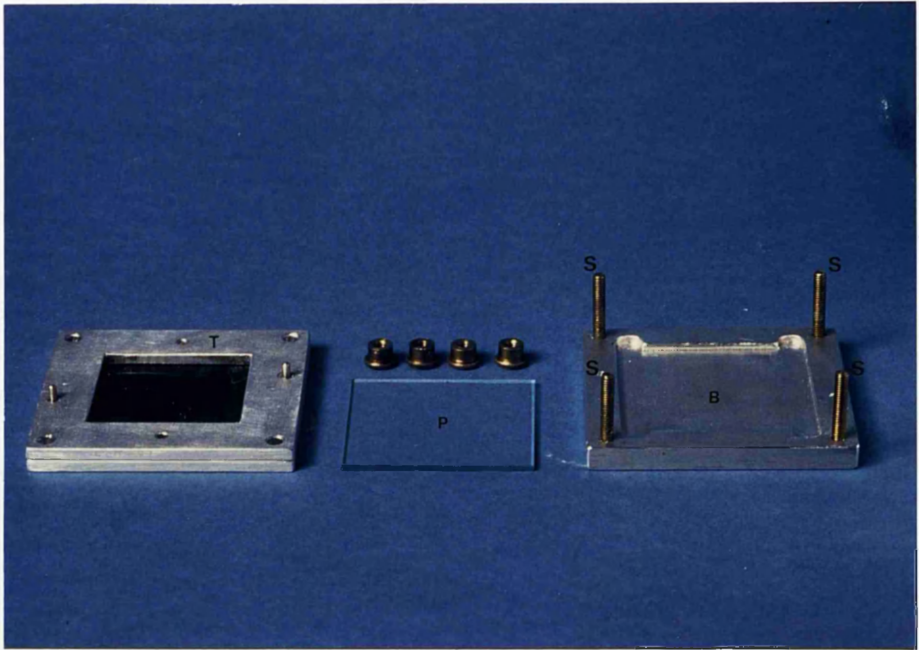

Figure 2.6 The microradiographic plate holder comprising the plate (P), the base (B), top (T) and locating screws (S).

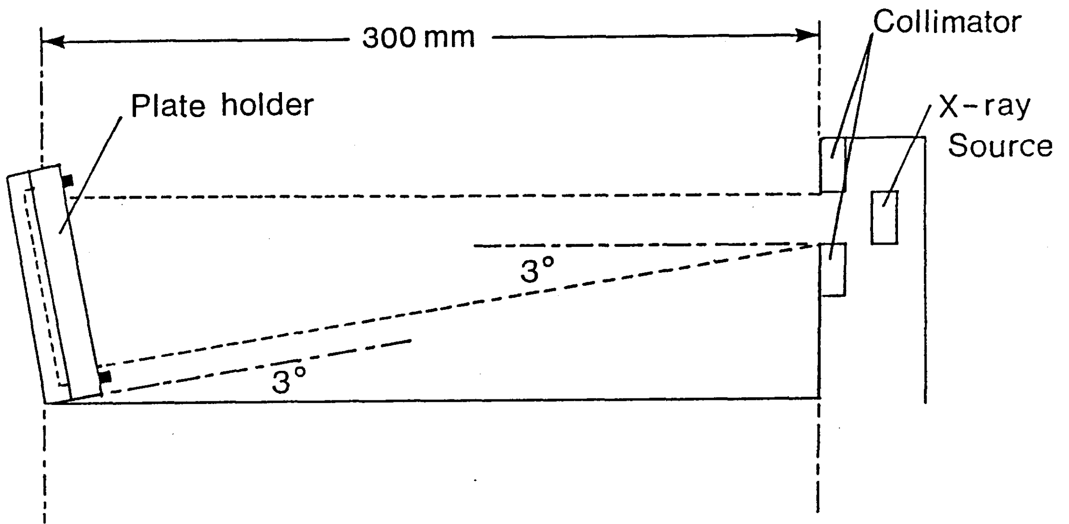

Figure 2.7 Diagrammatic representation of the radiographic apparatus, illustrating the angulation between the X-ray source and the plate holder.

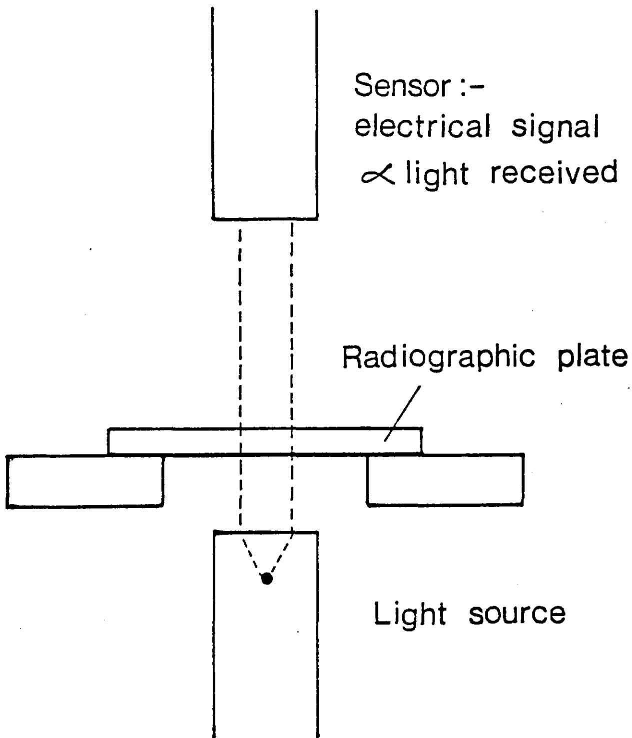

Figure 2.8 The principle of microdensitometry.

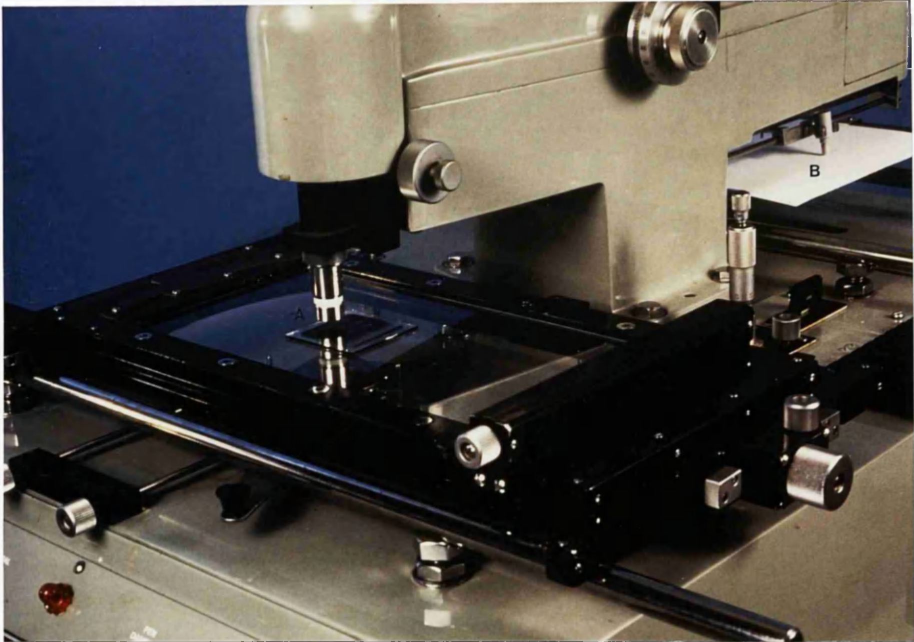

Figure 2.9 The Joyce-Loebl microdensitometer. A microradiographic plate is situated on the glass table (A) and to the rear of the apparatus, an analogue tracing (B) is being produced.

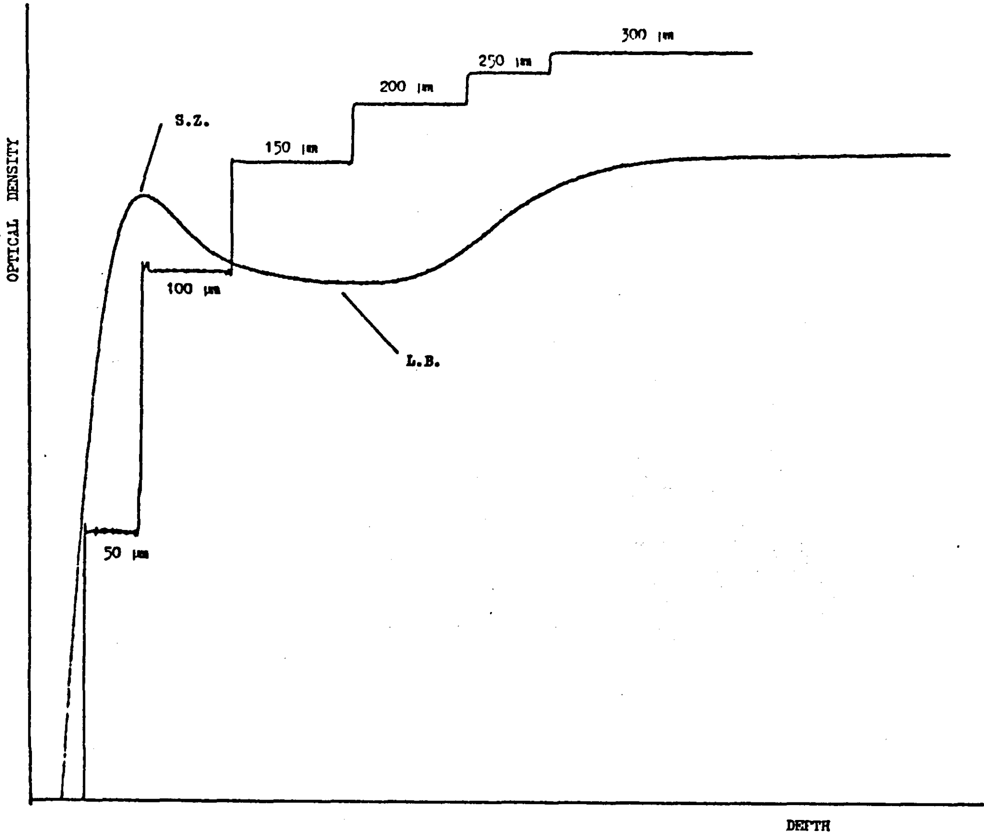

Figure 2.10 Analogue output tracing from the Joyce-Loebl of a typical enamel lesion and concommitant aluminium stepwedge. The surface zone (SZ), lesion body (LB) and normal enamel are seen.

Figure 2.11 The Leitz image analyser and the BBC-B microcomputer.

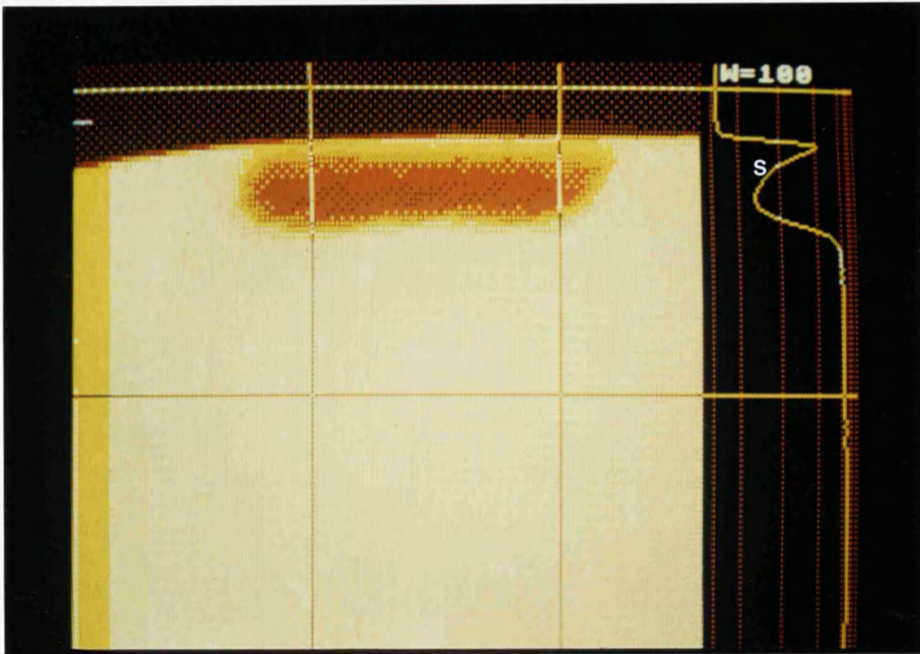

Figure 2.12 An enamel lesion, colour coded and as displayed on the BBC monitor. The grey levels of the aluminium stepwedge are represented by the red vertical lines on the right of the screen. The scan (S) has been taken from the area defined by the yellow vertical and horizontal lines, superimposed on the lesion.

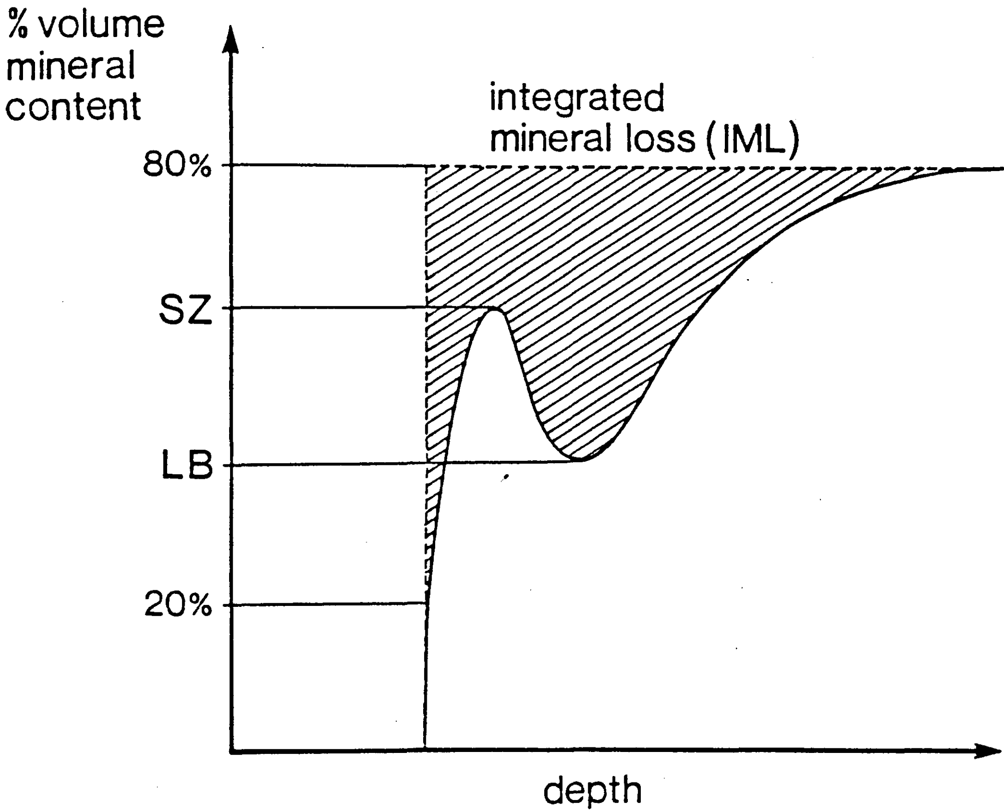

Figure 2.13 Typical microradiographic tracing illustrating the measured parameters:

1. % volume mineral content of the surface zone (SZ)
2. % volume mineral content of the lesion body (LB)
3. the integrated mineral loss (IML) - the shaded area.

CHAPTER 3 PRELIMINARY INVESTIGATIONS INTO MICRORADIOGRAPHIC REPEATABILITY AND LESION MINERAL CONTENT VARIATION

3.1 INTRODUCTION

As described in section 1.5, many caries remineralisation studies have lacked sensitivity because of the absence of a reliable control tissue. Several techniques have been employed for obtaining a control sample. Various workers (Featherstone *et al*., 1983; Retief *et al*., 1983; de Bruyn *et al*., 1985 a,b) have analysed specimens from the same tooth, half of which was used as control. Holmen *et al*. (1985 a,b), as stated previously in section 1.4.5, in their *in vivo* technique employed control specimens from different teeth within the same oral environment. The basic assumption for the above regimes is that the enamel does not vary in structure, mineral, organic or trace element content, not only within the same tooth, but from different teeth usually within the same mouth. Hallsworth *et al*. (1972) have shown that this assumption is unfounded and that enamel varies both from the natural surface to the amelodentinal junction, and from cervical to incisal aspects as far as mineral and trace elements are concerned. However, it was not until the *in vitro* studies of Featherstone & Silverstone (1982) and Harvey *et al*. (1982), that use was made of single sections, where baseline measurements from the test samples provided control values for subsequent comparison. In this technique the sensitivity to changes in mineral content

will depend on the repeatability of the measuring method. The variation and lack of homogeneity in both sound and carious enamel is a feature to which little research has been directed in recent years, other than the work of Hallsworth et al. (1972). The original mineral content of the enamel and fluoride concentrations as low as 0.12 ppm in a demineralising solutions are among some of the factors which are purported to influence both the rate and extent of demineralisation (Groeneveld et al., 1975; Borsboom et al., 1985). Also, Shellis (1984) concluded that the variation in caries prevalence between individuals and between contralateral teeth within the same mouth, could not be wholly explained by such factors as morphology, salivary composition and flow rate, but that there must be variation in the intrinsic susceptibility of the enamel.

The aims of the this part of the project were to investigate:

1. the repeatability of microradiographic/ microdensitometric measurements of enamel mineral content,
2. the variation in mineral content within a single area of demineralisation,
3. the variation in mineral content of areas of demineralisation in different sections from the same artificially produced carious lesion

-and-

4. the variation in mineral content of areas of demineralisation in sections taken from different

teeth exposed to the same acid gelatin for the same period of time.

3.2 MATERIALS AND METHODS

3.2.1 General

Six premolar teeth, extracted for orthodontic purposes and with no clinically obvious incipient lesions on their buccal surfaces, were used. All the teeth had recently erupted into the oral cavity. The enamel surface was cleaned with a rubber cup and a fine pumice-alcohol mixture to remove any calculus or pellicle deposits. The teeth were prepared and lesions created on their buccal surfaces, as described previously in section 2.2. Six or seven longitudinal sections were cut from each tooth and ground to a final measured thickness between 100 and 120 μm.

3.2.2 Microradiography and microdensitometry

The sections plus an aluminium stepwedge were mounted on Kodak high resolution plates Type 1A. Details of both the microradiographic and microdensitometric techniques have been outlined in section 2.5. Fig. 3.1 shows a typical microdensitometric tracing illustrating the measured parameters analysed during this part of the project.

Microdensitometric tracings of the lesions and the aluminium stepwedges were obtained using the Joyce-Loebl

3CS microdensitometer with a slit width of 10 μm (length 60 or 360 μm - vide infra) and the analogue tracings digitised into the computer, and mineral content values calculated as described in section 2.5.

3.3 REPEAT MEASUREMENTS

3.3.1 Method

To test the repeatability of the procedures, and hence the sensitivity of the single section technique, three lesions were each radiographed on five occasions. A micro-densitometric tracing through the central portion of the lesion was obtained from each microradiograph using a slit size of 360 x 10 μm.

Although the main part of the analysis in this chapter was carried out using the Joyce-Loebl 3CS Microdensitometer, it was also considered pertinent to test the repeatability of the measurement of mineral content using the Leitz ASBA image analyser.

3.3.2 Results

For the five repeat measurements A - E, the variations in mineral content of the surface zone (SZ) and the lesion body (LB) are shown in Fig. 3.2a and in Fig. 3.2b for the integrated mineral loss values. The standard deviations for the three lesions were 1.1, 1.2 and 1.4 (mean = 1.2) per cent volume mineral for the surface zone; 0.7, 0.8

and 1.3 (mean = 0.9) per cent volume mineral for the lesion body, and 1.93, 2.04 and 2.12 (mean = 2.12) μm for the integrated mineral loss respectively.

With regard to the ASBA unit, the variations were found to be within 1 %.

3.4 VARIATION ACROSS A LESION

3.4.1 Method

In order to test the variation in mineral content within a lesion, serial microdensitometric scans were obtained, by moving the region examined 100 μm towards the incisal extreme of the lesion between scans. A slit size of 60 x 10 μm was used. Two sections were analysed in this part of the study.

3.4.2 Results

The per cent volume mineral content of the surface zone, lesion body and the integrated mineral loss values, corresponding to different densitometric tracings, A - F or A - G (cervical to incisal), within the same lesion are shown in Fig. 3.3 for two lesions. The standard deviations of the surface zone values were 3.8 and 5.0 per cent volume mineral for the two lesions. The corresponding values for the lesion body and integrated mineral loss were 6.6 and 2.2 % volume mineral and 12.0 and 5.1 μm respectively.

3.5 VARIATION BETWEEN SECTIONS

3.5.1 Method

Eighteen sections from three teeth were used to study the variation in lesion mineral content between sections from the same tooth. Microdensitometric tracings of the central part of each lesion were obtained for each section using a slit size of 360 x 10 μm.

3.5.2 Results

The variations in mineral content measured in different sections (A - F or A -G) from the same tooth are shown in Fig. 3.4 (a,b) for three teeth. The demineralisation appeared less pronounced in those sections (C, D, and E) cut from the centre of the tooth. The standard deviation of the surface zone and lesion body values were 4.9, 4.8 and 8.4 (mean 6.0), and 3.3, 2.0 and 8.0 (mean = 4.0) % volume mineral respectively. The corresponding values for the integrated mineral loss were 11.0, 9.1 and 17.2 (mean 12.5) μm.

3.6 VARIATION BETWEEN TEETH

3.6.1 Method

Six teeth were exposed to the same batch of acidified gel under identical conditions for the same period of time. Microdensitometric tracings from the central portion of

the lesions were obtained using a slit size of 360 x 10 μm. The lesions studied were on the central longitudinal section from each tooth.

3.6.2 Results

Fig. 3.5 shows the variation in the measured parameters from tracings taken of sections cut from different teeth (A - F) exposed to the same acid gelatin for the same period of time. The standard deviations for the surface zone, lesion body and integrated mineral loss were 9.1%, 4.5% and 20.5 μm respectively.

The mean standard deviations for the three measured parameters are summarised in Fig. 3.6 for the four different studies reported. The variation in the parameters was smaller for the repeated measurements of the same lesion whereas the measurements for the areas of demineralisation taken from different teeth showed the greatest variation.

3.7 DISCUSSION

De Josselin de Jong and ten Bosch (1985) investigated the source of errors in microradiographic assessment of enamel mineral content and concluded that the microradiographic method was an accurate tool for investigating atomic concentration at the microscopic level. This part of the study has shown that microradiographic/ microdensitometric measurements of enamel mineral content were repeatable to

within an acceptable 2 - 4 % of the measured value, for both the Joyce-Loebl and ASBA units.

In agreement with Groeneveld _et al_. (1975) who employed hydroxyethylcellulose media (HEC) for creating lesions, this study showed large variations in mineral content in lesions created in different teeth exposed to the same acid attack. Hence, the use of a separate tooth as control will be less sensitive to small changes in mineral content as, for example, occurs during remineralisation.

In this study comparison was also made of mineral content variations in demineralised enamel from sections from the same tooth. Although, the variations were smaller than those found between teeth, they still demonstrated a considerable range of demineralised values when compared to repeat measurements on the same section. The apparently lower demineralisation in sections taken from the centre of a tooth requires further comment. It may be that there is an intrinsic variation in the susceptibility of enamel to acid attack. However, angulation of the enamel edge is more pronounced in sections further from the centre and this will have an effect both during the demineralisation process and on the microdensitometric measurement which will underestimate the mineral content of the surface zone to an extent depending on the angulation of the section. Whether or not this variation is true or apparant, the results indicate that those workers using separate sections from the same (non-abraded) tooth as control and test specimens, should ensure that sections are taken from

as near the centre of the tooth as possible to minimise differences between control and test specimens.

Although Groeneveld _et al_. (1975) reported a degree of homogeneity within lesions created using an HEC demineralising media, this was not the case here. In agreement with Crabb (1968), there was an observed variation in mineral content within a single lesion. This result is important, in that care is required when choosing the area of measurement within the lesion, especially in the single section technique where any misalignment of the measurement area between the baseline and later microradiographs will lead to a loss of sensitivity.

The smaller variations from repeat measurements on the same section imply that single section techniques which compare post-treatment measurements of mineral content with baseline measurements on the same lesion, will be more sensitive to changes in mineral content than techniques which use either a separate tooth or an adjacent section as a control specimen. This finding supports the first aim of the thesis, ie. to develop an _in situ_ technique for the use of single sections. As such, use was made of the single section technique in subsequent parts of the project.

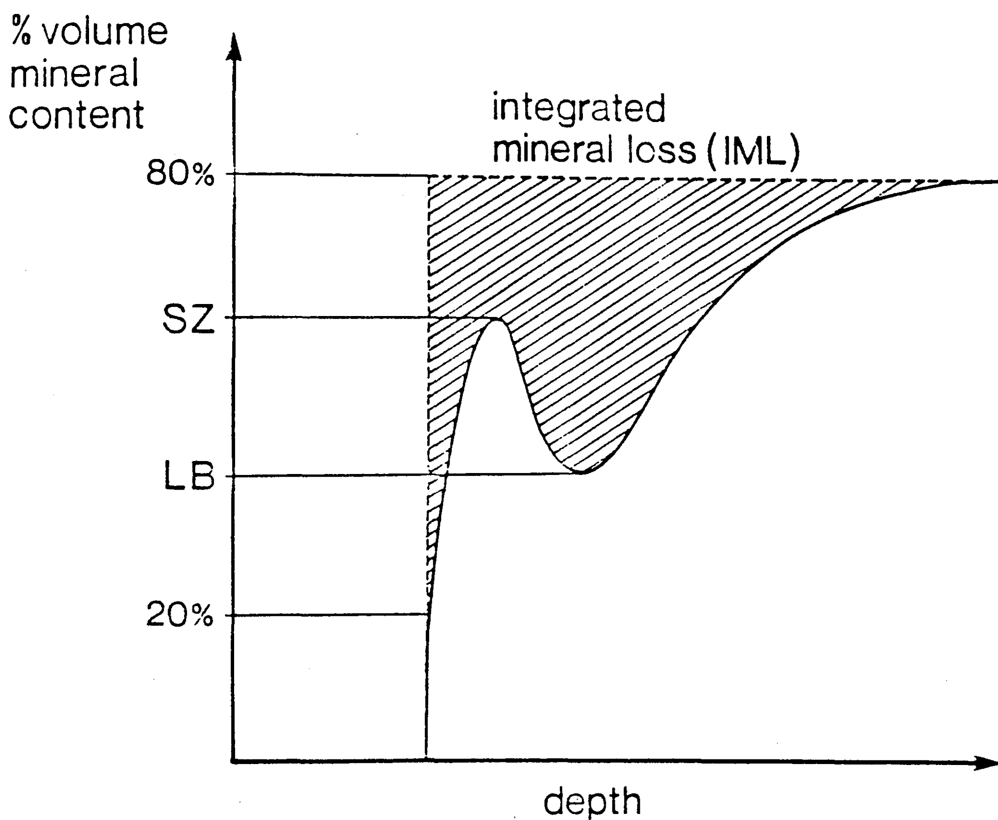

Figure 3.1 Typical microdensitometric tracing illustrating the measured parameters:

1. % volume mineral content of the surface zone (SZ)
2. % volume mineral content of the lesion body (LB)
3. the integrated mineral loss (IML) - the shaded area.

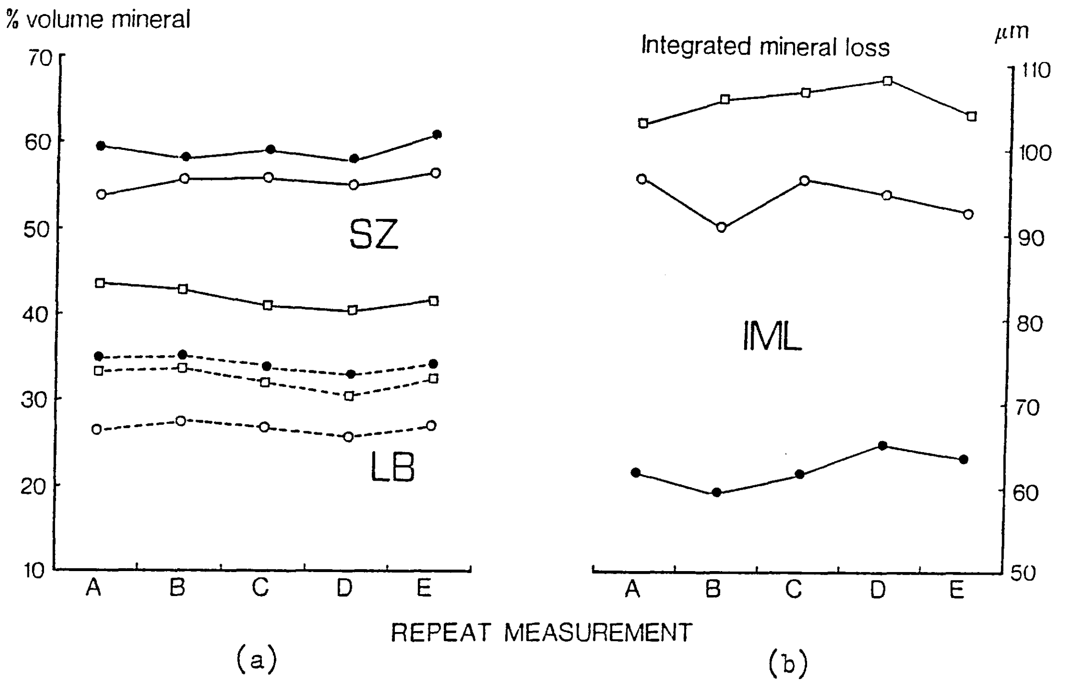

Figure 3.2 The variation in measured parameters of three lesions L1●, L2○ and L3□ with the repeat measurements (A - E). The illustration shows (a) surface zone (SZ) and lesion body (LB) and (b) the integrated mineral loss (IML).

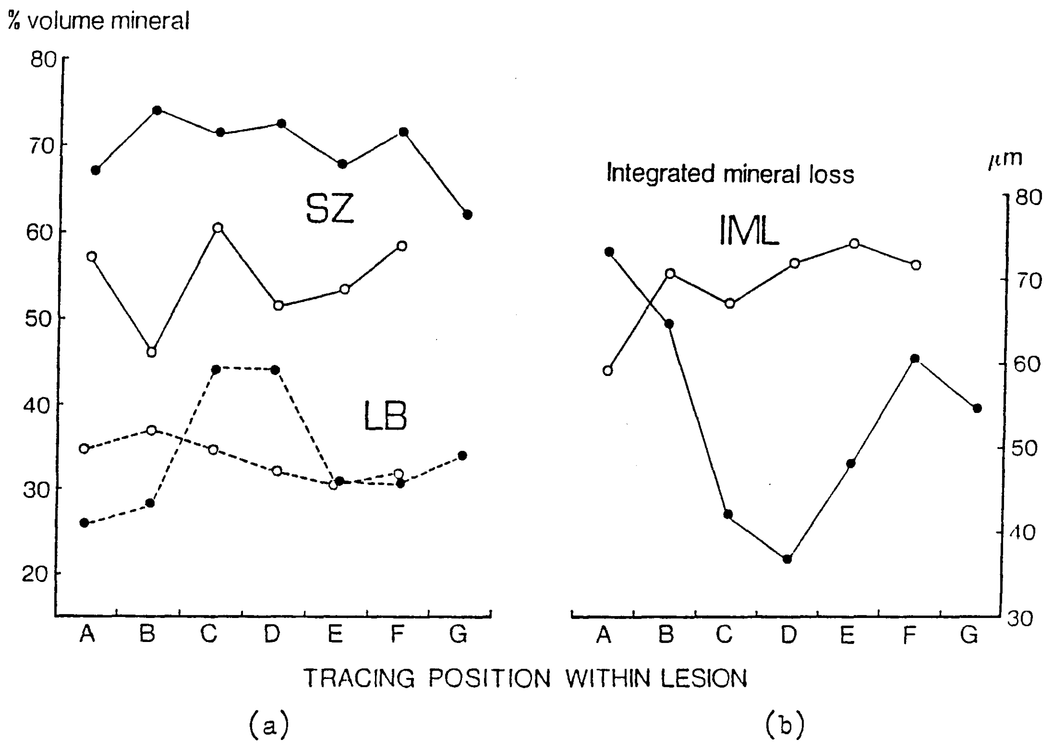

Figure 3.3. The variation in measured parameters for two lesions L1● and L2○, corresponding to different densitometric tracings (A - F or A - G) within that lesion.
(a) surface zone (SZ) and lesion body (LB) and
(b) integrated mineral loss (IML).

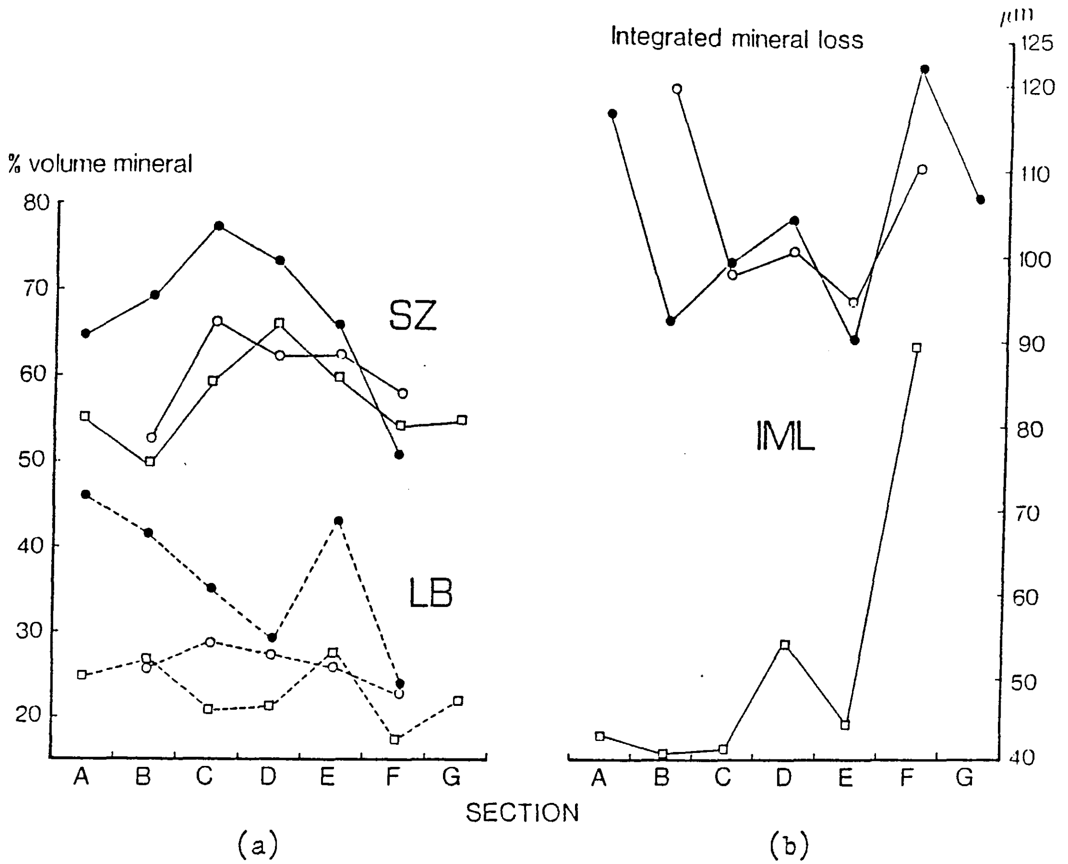

Figure 3.4 The variation in measured parameters from different sections (A - F or A - G) from the same tooth for three groups of sections (L1●, L2○ and L3□). (a) surface zone (SZ) and lesion body (LB) and (b) integrated mineral loss (IML).

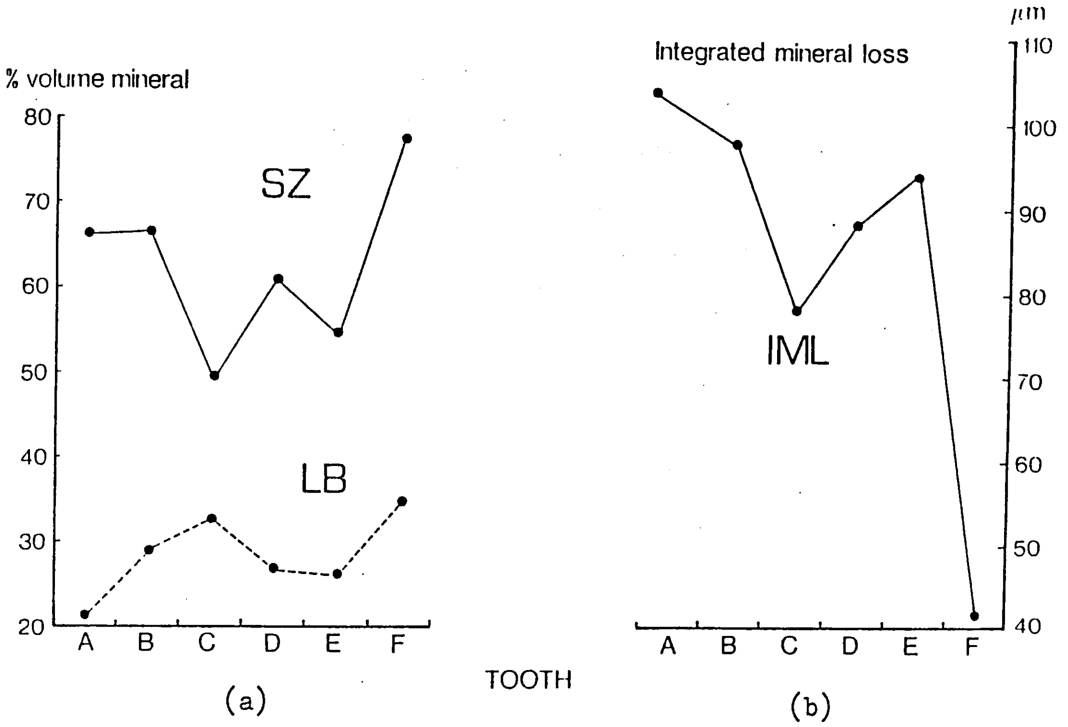

Figure 3.5 The variation in measured parameters of tracings taken from a single central section from different teeth (A - F). (a) surface zone (SZ) and lesion body (LB) and (b) integrated mineral loss (IML).

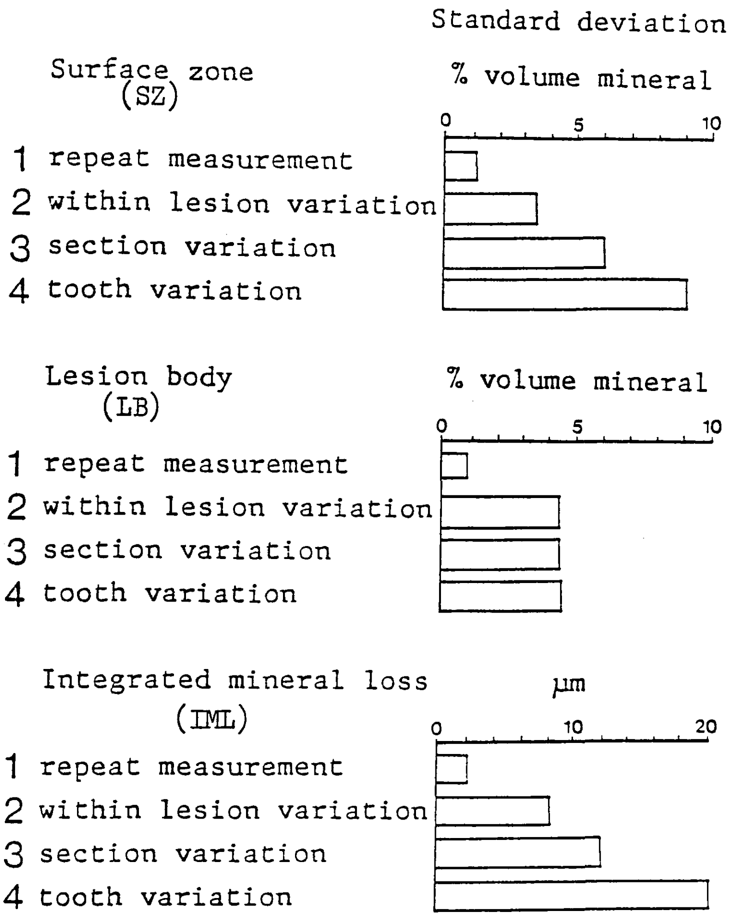

Figure 3.6 Mean standard deviations for the surface zone (SZ), lesion body (LB) and integrated mineral loss (IML), relating to Figs. 3.2 - 3.5.

CHAPTER 4 *IN SITU* STUDIES - APPLIANCE DESIGN AND VALIDATION

4.1 INTRODUCTION

The study of enamel de- and remineralisation in the natural oral environment, with its complex biological and biochemical interactions, has been the goal of many workers. Chapter 1 discussed in detail the various techniques which have been employed to investigate enamel remineralisation. It was concluded in section 1.5 that an *in situ* technique, where enamel lesions could be exposed to the oral environment and their mineral content subsequently measured at any point during the procedure, had inherent advantages. Furthermore, if such a method could be coupled with the use of single sections, the resultant model might be of importance in the investigation of remineralisation within the oral environment.

The aim of this chapter is to outline the design and construction methods of a new *in situ* appliance, suitable for the extension of the "single section" technique to the human mouth. In addition, it was important to establish if the plaque-like aggregates accumulating on the natural surface of the enamel specimens were similar to the adjacent interproximal area of the volunteer's natural dentition. The two methods used to test the composition of the deposits which formed on the appliance were

microbiological assessment, and acid/anion profile analysis using isotachophoresis. These tests were chosen since changes in organic acid levels of fasted plaque after sucrose exposure have been investigated thoroughly (Geddes, 1975; Gilmour _et al_., 1976; Distler & Kroncke, 1983, 1986; Geddes & Weetman, 1983) and the bacteria normally present in 7- to 9-day-old dental plaque have been well characterised (Ritz, 1967).

4.2 APPLIANCE DESIGN AND CONSTRUCTION

4.2.1 Requirements

The main objective was to construct an intraoral appliance on which enamel sections could be mounted and removed. Unlike previous _in situ_ techniques, the appliance would not require an edentulous space. That is, it would not necessarily link with the existence of a previous denture.

The main requirements were:

1. the design of the appliance should be such that saliva is permitted to freely contact any specimens mounted thereon; and that there should be a natural accumulation of plaque,
2. there should be no need for an edentulous space,
3. the device should be easily removed to facilitate the cleansing of the natural dentition,
4. any specimens mounted on the appliance should be easy to remove,

- and finally -

5. the appliance should be comfortable and easy to wear.

4.2.2 Construction methods

From upper and lower impressions taken in alginate impression material (Xantalgin, Bayer, Newbury, England), casts were poured in dental stone and any undercut areas on the lingual aspect of the mandibular arch blocked out using standard techniques. Opposite the $\overline{76|67}$ interproximal areas, small pieces of wax measuring 6 x 9 x 1 mm were placed on the cast, just inferior to the contact points of those teeth (Fig. 4.1 and 4.2). The cast and wax were then lightly warmed in tepid water and the wax was gently flattened on to the model using a glass microscope slide. The cast was duplicated and the appliance constructed from self-curing acrylic on the duplicate. This resulted in an appliance with two troughs, which had dimensions identical to the wax template on its fitting surface (Fig. 4.3 and 4.4). These troughs were to act as the experimental chambers on which enamel specimens would be mounted. To allow saliva to enter and leave the experimental chamber freely, an entrance and an exit were sited on the superior and inferior aspects of the trough respectively. Retention of the appliance was achieved by four half-round wrought stainless steel wire clasps (Fig. 4.3).

Before commencing these experimental procedures, approval was sought and granted from the Dental Ethics Committee of Greater Glasgow Health Board (Appendix III). Thereafter, each volunteer who participated in any of the appliance studies, was asked to wear the device for one week prior to commencing the experiment proper, to allow any rough areas or painful spots to be identified, and appropriately adjusted. In addition, a detailed protocol was formulated with regard to oral hygiene practice (Appendix IV). This ensured that the volunteers' natural dentitions were maintained in a plaque-free state, whilst leaving the chambers and specimens undisturbed.

Sections of enamel for use with the appliance were prepared as described previously (section 4.2) and, using x10 magnification, they were varnished with a proprietary nail-varnish (Max Factor, London, England) on all cut surfaces, leaving only the natural enamel surface exposed. After allowing the varnish to dry adequately, the specimens were cemented on to the base of the trough using the same varnish, as illustrated in Fig. 4.4. The sections were removed by dropping amyl acetate into the trough, thus allowing the varnish to be gently removed without damage to the specimens.

4.2.3 Salivary flow through the chamber

To ensure that saliva was flowing through the chamber, the appliance was worn for seven days, without specimens, and plaque allowed to accumulate within the chamber. A

plaque-disclosing tablet (En-de-kay, Stafford-Miller, Hatfield, England) was then taken for the prescribed 1 min, after which the volunteer was asked to rinse thoroughly. The appliance was then removed, and the plaque examined to check if it had become stained by the dye from the disclosing tablet. In all cases, it could be clearly seen that the plaque was brightly stained; thus, the tablet dye and saliva must have entered the trough freely during the 1 min period.

4.3 ASSESSMENT OF "APPLIANCE PLAQUE"

4.3.1 Methods and materials

Enamel sections were cut from premolar teeth, extracted for orthodontic purposes, as described in section 2.4. Artificial lesions had not been created on these teeth and from a close examination of the specimens, no incipient white spot lesions could be detected. The sections were, therefore, undemineralised. Each section was coated on all cut aspects, leaving only the natural enamel surface exposed. Four sections were subsequently mounted on the base of the appliance chamber of one volunteer with their non-varnished surfaces opposed, thus mimicing a "contact-point", as shown in Fig. 4.4. The volunteer was instructed to wear the appliance for 7 days during which the individual's normal dietary pattern was maintained and a non-fluoridated dentifrice used (Gibbs Dental Division, Port Sunlight, England). The appliance was removed briefly once per day to facilitate cleansing of the lower

lingual aspects of the natural dentition by brushing and flossing, apart from one interproximal area in the 76| region, as outlined in Appendix IV. Thus, plaque accumulation on the natural dentition was kept to a minimum although that developing in the experimental trough was left undisturbed. The procedure was repeated on four occasions for the acid anion profiles and on three occasions for the microbiological estimations.

4.3.2 Plaque sampling

At the end of the 7-day period, the volunteer was instructed to refrain from both late night snacks the day before and from breakfast on the morning of appliance removal, as this has been shown to exhibt the typical characteristics of starved plaque (Geddes, 1975). A sample of the 7-day-old plaque was taken for acid/anion profile estimations from the exposed enamel surface of one of the sections (Fig. 4.4) using a No. 11 scalpel blade and x 10 magnification, to minimise contamination of the specimen. A sample was also taken for isotachophoresis from the 76| natural interproximal region using a William's periodontal probe. Both samples were placed immediately on to the tops of two plastic containers, with some moist cotton wool in the base, and stored at 4°C. Within 10 min the sample was removed and placed into a preweighed plastic vial and reweighed, ensuring at least 1 mg wet weight of sample. Not later than 30 min from the time of sampling, the plaque was mixed with the leading electrolyte (_vide infra_) at 4°C, centrifuged at 20,000 g

for 15 min and stored at -20°C until analysed (Geddes & Weetman, 1983). The appliance was reinserted immediately after plaque sampling and the volunteer rinsed with 10 ml of a 10 per cent sucrose solution for 30 s. After a further 4 min the appliance was removed and two additional samples were taken from each of the natural and appliance sites. One pair of samples was for isotachophoresis as described earlier, while each of the second set of samples was placed into 1 ml of sterile anaerobic blood broth (Gibco-Europe, Paisley, Scotland) and used for microbiological assessment.

4.3.3 Microbiological methods

Plaque samples were dispersed by vortex mixing (Gallemkamp, East Kilbride, Scotland), followed by sonication for 15 s at a setting of 1.5 (Ultrasonic Sonicator, Heat Systems Ultrasonics, Plainview, N.Y.). Serial tenfold dilutions of plaque samples were immediately made from neat to 10^{-8} in anaerobic blood broth (Gibco-Europe, Paisley, Scotland). A standard inoculum (0.1 ml) of each dilution was inoculated on to each of the following media: Trypticase Soy Blood Agar supplemented with vitamin K and haemin (0.5 g per litre haemin - Gibco-Europe, Paisley, Scotland); Mitis Salivarius Agar supplemented with 20 per cent sucrose and 20 units bacitracin per 100 ml (Difco, Surrey, England), and Rogosa's Agar (Difco, Surrey, England). The inoculum was spread evenly over the surface of the agar using a right-angled sterile glass rod and the plates incubated

under anaerobic conditions for 4 days at 37°C. On average 15 min elapsed from the time of sampling to incubation of the plaque cultures. Plates with easily counted numbers of colonies were selected and the total bacterial, Lactobacillus spp. and Streptococcus mutans counts were calculated. All colonies from the blood agar plate which were selected for counting, and three colonies from each of the selective media plates, were subcultured on to blood agar and incubated until growth occurred. The isolates were identified using standard techniques, ie. morphology, growth conditions and biochemical tests. Streptococci were identified using A.P.I. Strep., (Basingstoke, Hampshire, England), anaerobic rods by Minitek (Becton-Dickinson, Oxford, England) and gram-positive rods by a combination of Minitek and acid end-product results. Finally, the percentage of each species in the original plaque samples was calculated. Details of the media used in this part of the project can be found in Appendices V - VIII.

4.3.4 Acid/anion methods

Acid/anions were analysed by isotachophoresis using an LKB 2127 Tachophor (LKB Instruments, Bromma, Sweden) fitted with conductivity and ultraviolet detection systems. The separation took place in a 610-mm Teflon capillary tube of 0.5 mm diameter maintained at a constant temperature of 12°C. The detection current was 50 μa.

The leading electrolyte was 5 mM hydrochloric acid adjusted to pH 4.2 by the addition of 6-amino-n-hexanoic acid, which acted as the buffering counter ion. Hydroxypropylmethylcellulose (0.2 per cent w/v) was added to the leading electrolyte to sharpen zone bounderies by reducing electro-endosmosis. The terminating electrolyte was 4 mM n-octanoic acid adjusted to pH 5.5 by the addition of 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris). All chemicals were purchased from BDH Chemicals Ltd., Poole, Dorset, England, except hydroxypropylmethylcellulose, which was obtained from Sigma Chemical Company Ltd., Poole, Dorset, England. Double-distilled deionised water was used to prepare the electrolytes.

Quantitative data were obtained by reference to standard solutions of lactic, acetic, propionic, succinic, pyruvic, formic and phosphoric acids (BDH Chemicals Ltd., Poole, Dorset, England). Statistical analyses were performed using a "t" test.

4.4 RESULTS

4.4.1 Microbiology

The microbial composition of plaque which formed on the appliance during the experiments was relatively consistent compared with the natural plaque which showed more variation (Fig. 4.5 and Table 4.1). Microorganisms not normally present in plaque, eg. candida, coliforms or staphylococci, were never isolated. In addition, S.

mutans was absent from both blood agar and Mitis Salivarius Bacitracin agar cultures, while lactobacilli were isolated on two occasions, one each from appliance and natural plaque samples, on the same run of the experiment.

4.4.2 Acid/anions

The results for the lactate, acetate and propionate profiles for both the appliance and natural plaques are listed in Tables 4.2, 4.3 and illustrated in Fig. 4.6. Statistical analysis showed that the lactic acid levels were significantly different after the sucrose rinse for both the natural ($p<0.001$) and appliance ($p<0.05$) plaques. The acetate and propionate levels were unaffected by sucrose rinsing. There were no significant differences in the levels of all acids tested between natural and appliance plaques, either before or after sucrose rinsing.

4.5 DISCUSSION

The acid anion results from this study are similar to those reported by Geddes (1975), Vratsanos et al. (1975) and Distler & Kroncke (1983, 1986), who have analysed pooled plaque from fasted subjects before and after sucrose rinsing. In the present study, like those cited above, it was found that the lactic acid exhibited a steep increase following sucrose exposure. The concentrations of pyruvate, formate, phosphate and succinate were present in minor amounts. Similar findings have been reported

previously (Geddes, 1984), therefore any major differences between the natural and appliance plaques would have resulted in more evident changes in the levels of the lactic, acetic and propionic acids.

It is well known that the microbial composition of dental plaque varies considerably at different sites on the same tooth, and at the same site on different teeth (Marsh & Martin, 1984). In addition, the composition of dental plaque is known to fluctuate on the same tooth and site with time. According to the microbiologist with whom this part of the work was undertaken, the qualitative and quantitative variations which occurred on the enamel of the *in situ* appliance and natural plaque samples are acceptable. Furthermore, the composition of the deposits which formed on the *in situ* enamel was within the range normally accepted for dental plaque collected 7-9 days after thorough prophylaxis (Ritz, 1967).

The results of both the acid anion profiles and the microbiological estimations therefore suggest that the deposits which accumulate on the exposed enamel surfaces mounted on this new intraoral device, have both microbiological and biochemical properties similar to natural dental plaque.

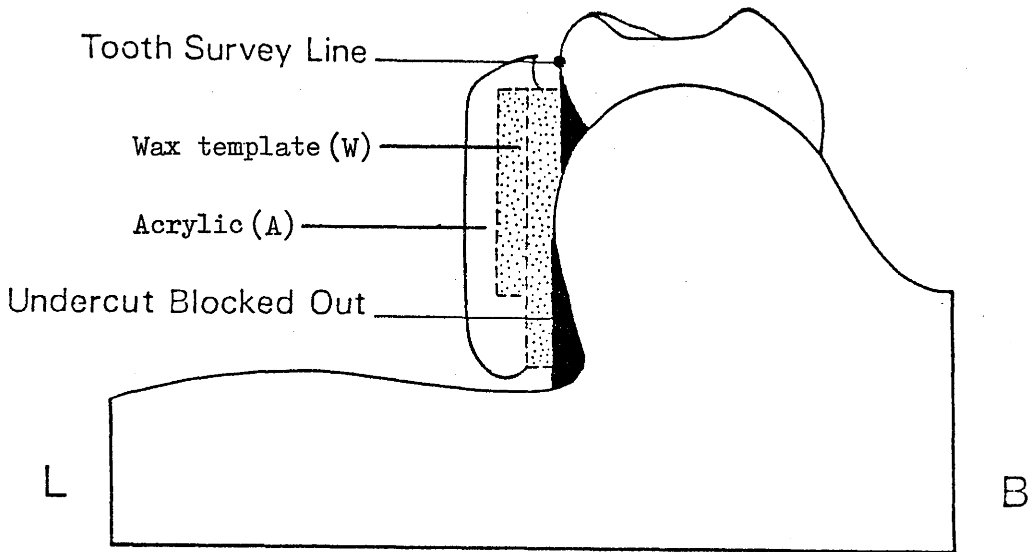

Figure 4.1 Diagramatic representation of a cross-section through the contact-point between the first and second mandibular molars, showing the lingual (L) and buccal (B) aspects of the cast. Situated on the lingual aspect of the cast are the wax template (W) and the acrylic of the appliance (A).

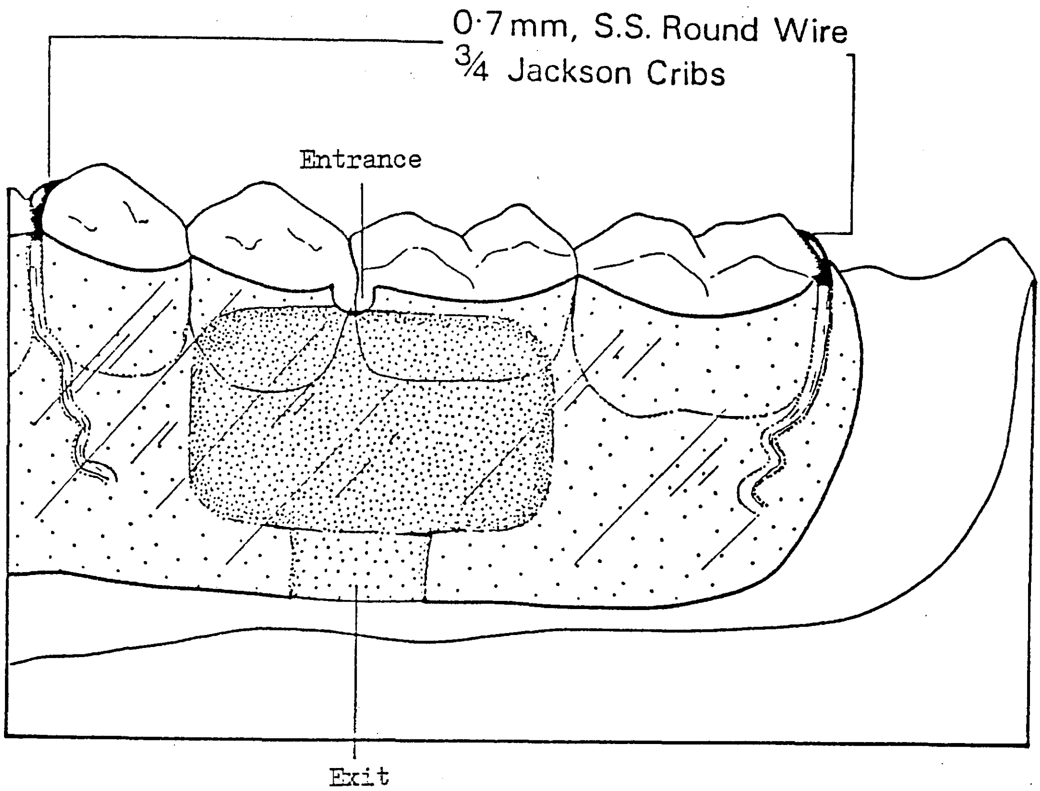

Figure 4.2 Diagramatic representation of the mandibular appliance <u>in situ</u> , showing the entrance and exit into the enclosed experimental trough.

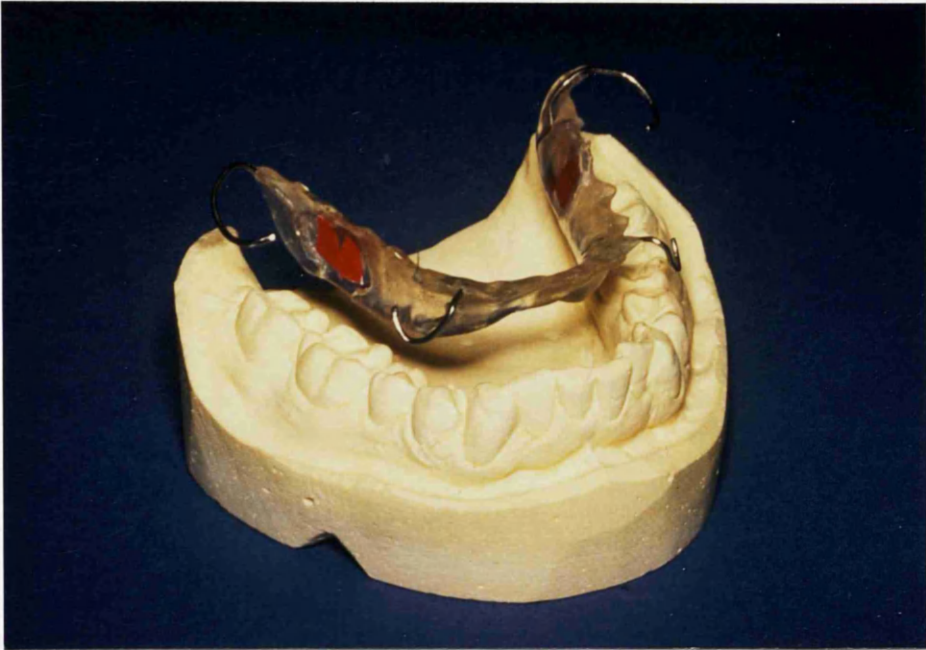

Figure 4.3 Full view of appliance suspended over cast of mandibular teeth, illustrating sections mounted on the right experimental trough. The stainless steel retaining clasps are evident.

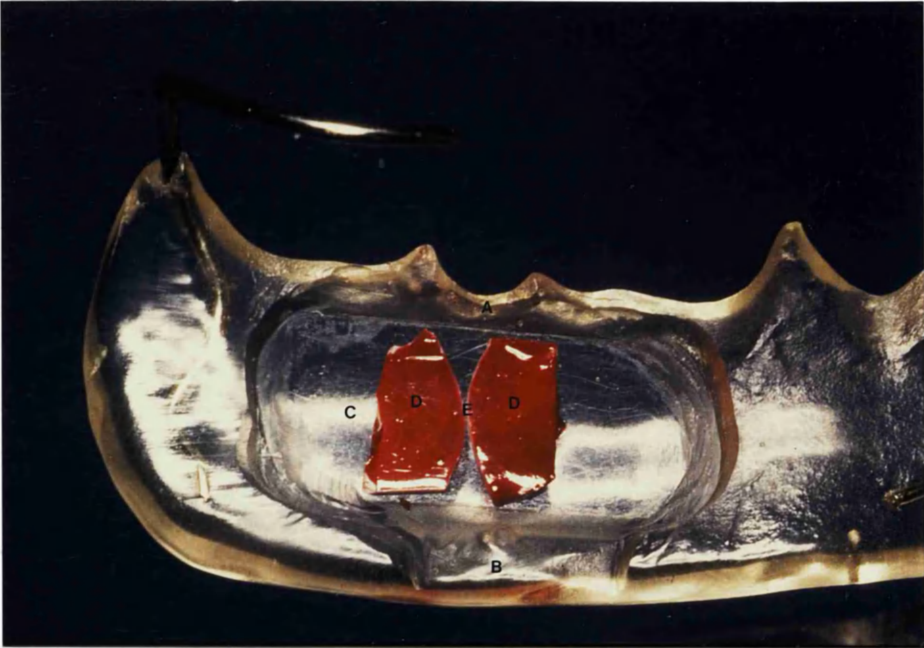

Figure 4.4 One side of the appliance showing the entrance (A) and the exit (B) to the trough (C). Enamel sections (D) are mounted in a 'contact point' situation (E).

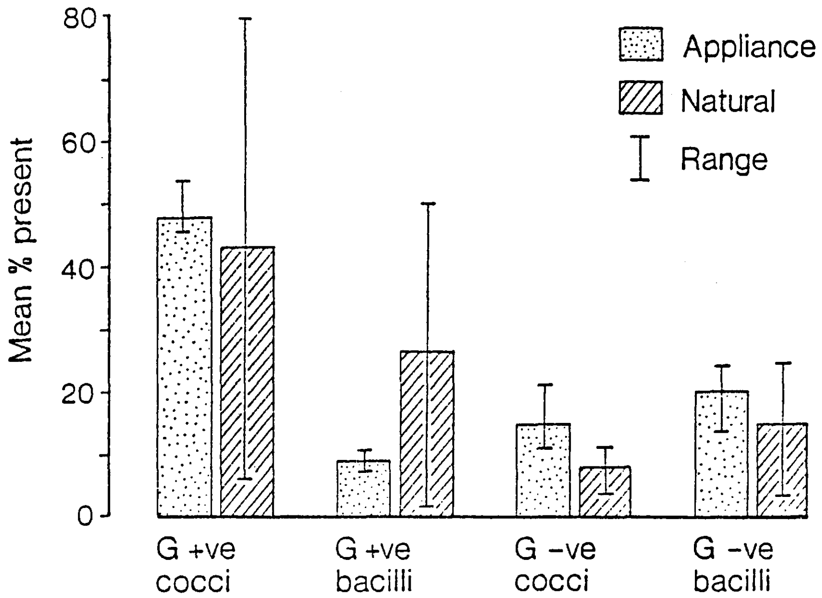

Figure 4.5 Histogram of the predominant cultivable flora from 1-week-old 'appliance' and natural plaque grouped into positive and negative gram species.

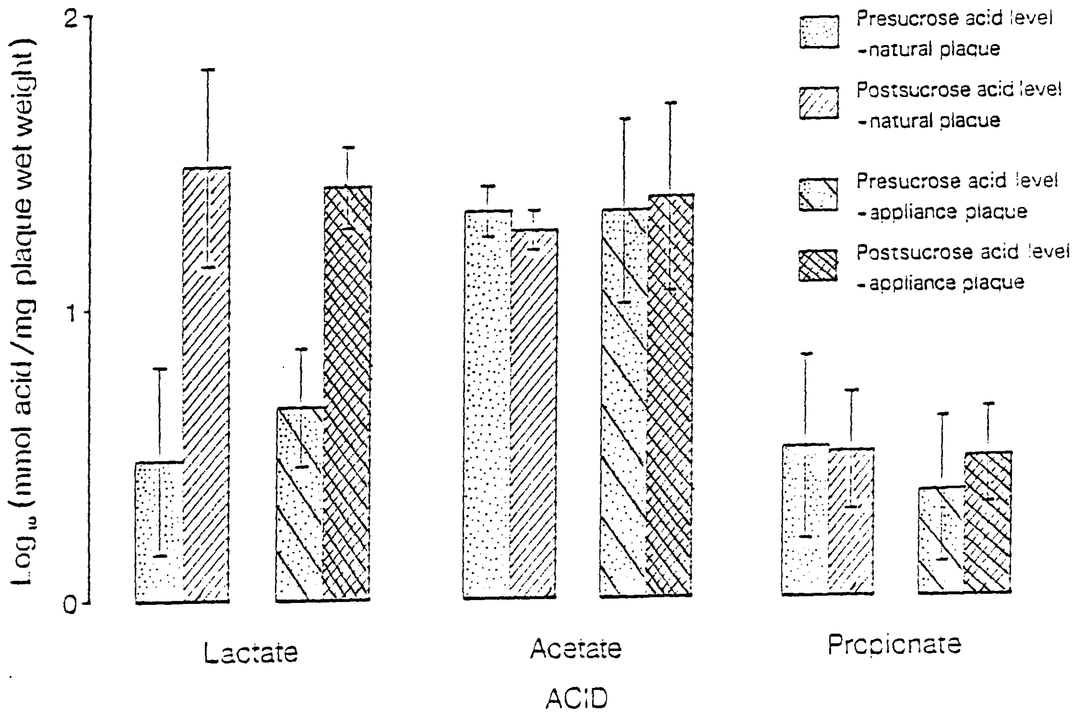

Figure 4.6 Logarithm to the base 10 (± 1 S.D.) of the various acid/anion concentrations (n.mol./ mg wet weight plaque) from the appliance and natural plaque samples, before and after a 10 % sucrose rinse.

Table 4.1

The predominant cultivable flora from 1-week-old "appliance" and natural plaque

	Mean % of total cultivable flora		Range, %	
	appliance	natural	appliance	natural
Streptococcal spp.	48.5	43.3	47.2-53.2	6.2-80.1
Gram-positive rods (predominantly Actinomyces)	9.0	27.0	10.9-12.4	2.2-51.9
Gram-negative rods (predominantly Bacteroides)	8.7	5.7	7.0-10.0	0.1-11.3
Veillonella spp.	15.4	7.9	12.5-21.8	4.3-11.4
Fusobacteria spp.	5.1	6.3	0.9- 8.0	2.2-10.1
Capnocytophaga spp.	6.0	3.3	5.3- 6.4	1.4- 5.1
Individual species				
Streptococcus sanguis type 1	6.2	0.0	0.0-18.6	0-0
Streptococcus sanguis type 2	25.6	14.6	21.3-29.0	2.5-25.7
Streptococcus mitior	5.3	0.0	0.0-12.0	0- 2.2
Actinomyces odontolyticus	3.5	0.8	0.0-10.6	0- 1.5
Actinomyces naeslundii/viscosus	0.7	22.1	0.0- 1.3	0.0-46.8
Bacteroides intermedius	2.3	0.0	0.3- 4.0	0-0
Anaerobic streptococci	10.8	28.8	0.0-18.2	3.7-52.3

Table 4.3

Acid/anion concentrations (nmol/mg wet weight) from 1-week-old natural plaque before and after a 10% sucrose rinse

Natural	Lactate		Acetate		Propionate	
	before	after	before	after	before	after
Sample 1	2.9	48.6	42.9	48.1	5.2	4.8
Sample 2	2.7	31.2	24.1	25.8	1.6	3.6
Sample 3	4.0	23.3	28.9	34.0	5.5	6.9
Sample 4	4.2	28.3	8.6	12.7	1.6	3.8
Sample 5	9.4	23.0	33.1	32.9	6.9	3.3
Sample 6	4.2	16.6	8.2	6.8	1.4	2.1
Mean	4.6	28.5	24.3	26.7	3.7	4.1
SD	+ 2.2	+ 10.1	+ 12.6	+ 13.8	+ 2.2	+ 1.5

Table 4.2

Acid/anion concentrations (nmol/mg wet weight) from 1-week-old appliance plaque before and after a 10% sucrose rinse

Appliance	Lactate		Acetate		Propionate	
	before	after	before	after	before	after
Sample 1	2.8	53.0	15.5	16.9	3.0	4.4
Sample 2	13.7	74.0	27.2	26.5	13.1	9.0
Sample 3	3.1	48.0	23.4	17.2	1.9	2.8
Sample 4	1.5	12.8	22.3	19.8	5.2	3.8
Sample 5	8.0	21.3	16.1	19.9	2.1	1.9
Sample 6	1.4	12.7	22.2	19.9	5.2	4.0
Mean	5.1	37.0	21.1	20.0	5.1	4.3
SD	+ 4.5	+ 23.0	+ 4.1	+ 3.2	+ 3.8	+ 2.3

CHAPTER 5 IN SITU STUDIES - REMINERALISATION

5.1 INTRODUCTION

Fluoride containing dentifrices are widely used as anticaries agents (Little et al., 1971; Mellberg, 1980) and are now thought to have made a major contribution to the general decline in caries experience over the last 10 to 15 years (Nikiforuk, 1985). Whilst the epidemiological data from clinical caries trials points unequivocally to the benefits of a fluoridated dentifrice when compared to a non-fluoridated placebo (Andlaw & Tucker, 1975; DePaola, 1983; Mellberg & Ripa, 1983; Stookey, 1985), the precise mechanism of action of the fluoride ion in the carious process has yet to be ellucidated. There is general agreement, nevertheless, that the primary mode of action of topical fluoride is on the incipient enamel lesion rather than normal enamel (ten Cate & Arends, 1977; Gelhard, 1978; Koulourides & Cameron, 1980; Featherstone et al., 1982; Mellberg & Chomicki, 1983; Mellberg & Mallon, 1984).

Dentifrices are used as an adjunct to the cleansing of the oral tissues and it has been reported that dentifrices containing NaMFP enhance the remineralising properties of saliva (Naylor & Glass, 1979). Thus, it has been postulated that if the influence of fluoride on the early

enamel lesion mineral redeposition is the primary cause of the decrease in caries prevalence, then it is theoretically possible to increase that remineralising ability by "improving" the dentifrice (Featherstone *et al.*, 1982).

Of the topical fluoride agents, fluoridated dentifrices are the most commonly available for caries prevention (Horowitz, 1983; Mellberg & Ripa, 1983). Thus, dentifrices were chosen as the fluoride vehicle to be tested. Thus, the aim of this part of the project was to evaluate the remineralising abilities of six dentifrices, these having three concentrations of NaMFP, two concentrations of NaF and a non-fluoridated placebo, using the *in situ* appliance developed in this study. As stated in Chapter 1, there is confusion as to the most effective means of delivery, and the concentration and frequency of fluoride, which would result in the optimum remineralising ability of a fluoride compound.

Results of recent *in vivo* remineralisation experiments involving dentifrices have been mixed and often contradictory. Featherstone *et al*. (1982) and Mellberg *et al*. (1985), have reported significant differences between test and control dentifrices. In the former study where volunteers were asked to brush twice daily with either a 0.76 % NaMFP dentifrice or a non-fluoridated control, the authors reported significant differences

between test and control pastes. In the latter, greatest remineralisation was found to exist in those lesions of greatest demineralisation. This suggested that there should be careful selection of the lesions chosen for a study, and this aspect is considered in greater detail in succeeding parts of this chapter. In both studies, however, great person-to-person variation was noted, with some subjects remineralising artificial carious lesions to a greater extent than others. This theme was extended by Corpron et al. (1986) who noted similar variation between subjects, but also concluded that those subjects who appeared to remineralise better were also best at resisting further mineral loss by acid exposure.

Smits & Arends (1985) and ten Cate & Rempt (1986), however, failed to demonstrate any significant differences in remineralisation ability between a fluoridated dentifrice and a placebo. Smits & Arends (1985) suggested that the variation in mineral content of the enamel slabs used may have been large enough to mask any minor differences. Ten Cate & Rempt (1986) stated that either mineral uptake and loss may have been in balance, or that the plaque overlying the slabs was in equilibrium with the enamel. Whilst accepting the personal variation between subjects, as witnessed by others, ten Cate & Rempt (1986) suggested, with little evidence, that the latter was the more likely.

From an epidemiological point of view, the results of clinical toothpaste trials have had mixed success. In the first instance, the caries protective role of topically applied fluoride from dentifrices, when compared with a nonfluoridated placebo, is unequivocal. However, when attempts have been made to ellucidate a concentration effect on the prevalance of carious lesions, a relatioship is less easily demonstrable. A number of workers have claimed no such association (Forsman, 1975; Koch, 1982; Mainwaring & Naylor, 1983; Juliano et al., 1985; Ripa et al., 1987). The majority of those who have demonstrated a concentration effect, have employed dentifrice fluoride levels ranging from 250 ppm to 1500 ppm (Hodge et al., 1980; Buhe et al., 1984). However, one notable exception is the clinical trial of Stephen et al. (1987), where an apparent concentration effect has been demonstrated when comparing fluoride concentrations from NaMFP of 1000 ppm to 2500 ppm. The findings of Stephen et al. (1987) are in conflict with those of Ripa et al. (1987) who compared dentifrices of not dissimilar fluoride concentration. However, the reported compliance of the subjects from the clinical trial of Ripa et al. (1987) was poor (48 % at 2 years) as were their methods of client persuasion and monitoring. In addition, no radiographs were taken in the Ripa et al. (1987) study, which could only reduce considerably the trial's sensitivity to discriminate between test products. Thus, the conclusions from that work should be treated with some scepticism. There is continuing discussion, nevertheless, on the optimum dentifrice fuoride concentration which will

produce a maximum caries preventive benefit.

The first part of this chapter is concerned with an assessment of the salivary and dental profiles of those individuals who participated in this part of the project. It was, thus, considered important that this be carried out before embarking on subsequent remineralisation experiments, to ensure that all subjects were within acceptable limits.

5.2 METHOD - INDIVIDUALS' PROFILES

5.2.1 Salivary flow

Each of the volunteers was asked to chew a paraffin wax capsule (Orion Diagnostica, Helsinki, Finland) and spit into a calibrated specimen container for 5 min, during which they were asked not to speak nor swallow saliva. At the end of this period, the volume of saliva was assessed.

5.2.2 Buffering capacity

To test the salivary buffering capacity, 1 ml of saliva was added immediately to a Dentobuff salivary kit (Orion Diagnostica, Helsinki, Finland) which has a scale of pH 3.5 - 9.0. Two tests were performed from each volunteer's saliva to ensure reproducibility of the test.

5.2.3 Calcium/phosphate analysis

An analysis was made of the salivary calcium and phosphate levels using isotachophoresis in a similar fashion to that carried out for the acid/anion analyses described in Chapter 4. In addition to a change in polarity, calcium analysis involved changing both the leading and terminating electrolytes to 10 m.mol potassium acetate, adjusted to pH 7.4, and 10 m.mol cadmium chloride respectively (Everaerts et al., 1973).

5.2.4 DMFS & DMFT

A clinical assessment of each of the volunteers' DMFT and DMFS scores was made by the author using mirror, probe (0.5 mm diameter) and standard dental chair lighting.

5.2.5 Calculus

Calculus was assessed using the method of Volpe & Manhold (1963) employing a graduated Williams periodontal probe to assess the extent of calculus growth on the lingual aspects of the lower six anterior teeth.

5.2.6 Salivary fluoride levels

Volunteers were provided with a supply of 0.76 % NaMFP (1000 ppmF) dentifrice and asked to use only that dentifrice for 7 days. A sample of saliva was taken at the end of the one week period late in the afternoon,

ensuring at least 6 hrs since last brushing. This level was taken to be the baseline. Each subject was then asked to brush for a 2 min period using the same dentifrice and after a further 5 min period a second salivary sample was donated. One volunteer (subject B) acted as the control and had used a non-fluoridated dentifrice. The fluoride levels were assessed using a selective ion electrode and Ionalyser Microprocessor (Orion Research, Massachusetts, U.S.A.).

5.3 RESULTS - INDIVIDUALS' PROFILES

Figures 5.1, 5.2 and 5.3 illustrate the results for volunteers' salivary flow, buffering capacity and calcium/ phosphate analyses. Clearly, there was no great variation between each subject, as was the situation for the DMFT/DMFS, calculus scores and salivary fluoride levels, detailed in Table 5.1. Although the fluoride levels showed greater variation, they were still considered within acceptable limits. As the aim of these studies was to determine if any of the volunteers should be excluded from the remineralisation experiment, since no subject exhibited results which were considered abnormal, none was eliminated.

5.4 METHODS AND MATERIALS - REMINERALISATION

5.4.1 General methods

Five volunteers were employed in this experiment, all of whom worked in the Glasgow Dental Hospital. An _in situ_ appliance, as described previously in Chapter 4, was constructed for each volunteer. Sections were prepared of unabraded human premolars, on which artificial carious lesions had been created previously (Chapter 2). For each experimental run of 5 weeks' duration (_vide infra_), four specimens were mounted on to the troughs of each appliance. Subjects were provided with a copy of the "Instructions to Appliance Wearers" regarding the practising of oral hygiene (Appendix IV), and were asked not to rinse after dentifrice use (Featherstone _et al_., 1982). On each occasion, subjects were provided with the dentifrice to be tested at least one week prior to appliance insertion. Each was asked to wear the appliance for 7 days (ie. 14 dentifice exposures). Sections were removed from the appliance by dropping amyl acetate into the troughs and allowing the sections to "float" free. They were then taken through two concentrations of alcohol and eventually into distilled water. Microradiography of the sections was then carried out, as detailed in Chapter 2. Subsequently, they were remounted on to the appliances and returned to the volunteers. The subjects were asked to continue using the test dentifrices when not participating in the test, during the 2 - 3 days necessary

to remove the specimens, carry out microradiography and replace them on to each of the appliances. This ensured that baseline salivary fluoride levels would not be altered by using a different dentifrice.

The dentifrices employed were provided by Unilever Research (Gibbs Dental Division, Port Sunlight, England). The three NaMFP dentifrices were concurrently being tested on a large scale epidemiological trial, involving 3000 schoolchildren in the Lanarkshire area, as previously discussed (Stephen et al., 1987). The fluoride content of each was as follows:

Nonfluoride		Nonfluoride	
1000 ppmF	NaMFP	1000 ppmF	NaF
1500 ppmF	NaMFP	1500 ppmF	NaF
2500 ppmF	NaMFP		

Details of the dentifrice formulations are listed in Appendix IX. For all runs of the experiment appliances were returned at the end of each week, as stated previously. However, for runs 3 and 4 it was decided that weekly microradogography was unnecessary. Thus, in those two runs, microradiography was carried out at baseline, and after 2 and 5 weeks' exposure. This had the additional advantage of minimising section handling. In all cases, plaque and debris were removed from the sections at weekly intervals.

5.4.2 Experimental protocol

The five volunteers were divided into two groups of three and two subjects and new sections were produced for each run of the experiment. In group 1, each volunteer used all three NaMFP dentifrices and the placebo for each experimental period of 5 weeks, whereas the volunteers of group 2 used each of the NaF dentifrices and the placebo, again for 5 weeks. The volunteers were "blind" as to which dentifrice they used since each paste was identically packaged and marked only with a code. The general plan of the experimental protocol is detailed in Table 5.2, illustrating the cross-over arrangement employed. Unfortunately, the non-fluoridated dentifrice of identical flavour to each of the test dentifrices did not become available until each experiment was well underway. Thus, it was decided to perform the placebo run at the final stage in the experiment. Before commencing tha placebo run, the non-fluoridated dentifrice was distributed to the sujects at least 2 weeks prior to appliance wearing.

5.4.3 Method of analysis

For each volunteer the change in lesion integrated mineral loss (IML) was plotted against time and a "least squares fit" carried out on the data. An example of the procedure is illustrated in Fig. 5.4, where the solid lines represent the IML analysis pertaining to a single lesion, and the dotted line is the least squares fit to all the

lesions. For clarity, in that example, there are only four lesions illustrated. The number of analysable lesions was determined by two factors. Firstly, only those lesions which lasted the 5 weeks without any evidence of damage were considered for analysis. Secondly, a least squares fit was carried out solely on lesions within a predetermined initial IML range (vide infra). The resultant gradient or slope is a measure of the remineralisation rate for that individual when using one particular dentifrice. It is this value, with its appropriate standard error, which is employed when comparing the change in remineralisation rates for the different fluoride concentrations. Thus, for each volunteer there are four and three remineralisation rates (± 1 S.E.) for the NaMFP and NaF dentifrice groups respectively. In addition, a least squares fit was carried out on the changes in IML -vs- time for each individual lesion. Similar analyses were carried out on the changes in mineral deposition within the surface zone and the lesion body of each lesion analysed.

5.4.4 Statistical Analyses

An analysis was carried out within each subject on all 3/4 dentifrices tested using a one-way-analysis of variance (ANOVA). If any significant differences were evident, each paste was compared in a pair-wise manner using a multiple comparison test (Scheffe S Test).

5.5 RESULTS I - EFFECT OF LESION SIZE

In a parallel *in situ* study using the 1000 ppm NaF dentifrice (Strang *et al*., 1987), it was noted that the remineralisation rate for an individual lesion increased linearly with the initial IML. Thus, it was decided to analyse the data obtained in this project in a similar manner: (a) to investigate if there was a lesion size effect evident with the other dentifrices and, (b) to select out only those lesions within a certain range for inclusion in the main analysis. Fig. 5.5 shows an example of the data from one volunteer. Clearly there is an increase in the remineralisation rates of the lesions as the initial IML increases. This was the case with both the NaMFP and NaF dentifrices, although as illustrated in Figs. 5.6 & 5.7, no such association was demonstrable with the nonfluoridated placebo. Thus, as stated in section 5.4.2, it was decided that the initial IML's of those lesions analysed ought to be within a similar range. The means (± 1 S.E.) detailed in Table 5.3, were within a similar range after selecting out several lesions in some instances. In most cases a compromise had to be achieved by balancing the initial IML's while still retaining an acceptable number of lesions for analysis.

5.6 RESULTS II - THE SODIUM MONOFLUOROPHOSPHATE DENTIFRICES (NaMFP)

5.6.1 Summary

For the IML data, two of the three volunteers (B & C) exhibited significantly increased remineralisation with the fluoridated dentifrices as compared to that of the non-fluoridated placebo. No significant differences were apparent between the fluoridated dentifrices (see Table 5.4 for significance levels). There were no significant differences between any of the pastes with volunteer A. Analyses of the changes in surface zone mineral content from volunteer C, however, showed a clear concentration effect between the fluoride content of the dentifrice and the amount of mineral deposited in that region of the lesion.

Figures 5.8 and 5.9 illustrate two examples of densitometric scans of two lesions at baseline and after 5 weeks' exposure to the oral environment when employing the 2500 NaMFP dentifrice. Clearly, there is a noticeable increase in the mineral content in both lesions' surface zones and, to a lesser extent, in the lesion bodies. The changes in IML values for lesions A and B were 5.5 and 5.8 respectively.

5.6.2 Integrated mineral loss changes

Figures 5.10 - 5.12 illustrate the combined remineralisation data from all four dentifrices tested, for all analysable lesions for each of the three volunteers. Figures 5.13 - 5.18 demonstrate examples of the least squares fit to one volunteer, as an example, and the combined data for each NaMFP dentifrice concentration.

Analysing the change in IML, only two of the three volunteers using the NaMFP dentifrices and the appropriate non-fluoridated placebo, showed significantly better remineralising properties when using each of the fluoridated dentifrices. An analysis of the data from volunteer B (Fig. 5.11), showed the non-fluoride dentifrice was significantly different from the 1000 ppm ($p<0.01$), the 1500 ppm ($p<0.05$) and the 2500 ppm ($p<0.05$) NaMFP dentifrices. However, when comparing the remineralising rates of each fluoridated dentifrice, none was significantly different ($p>0.05$). A similar set of data was obtained from volunteer C (Fig. 5.12), showing significant differences between the non-fluoride dentifrice and the 1000 ppm ($p<0.01$), the 1500 ppm ($p<0.01$), and the 2500 ppm ($p<0.01$) NaMFP dentifrices. No significant differences were detected between each of the fluoridated dentifrices ($p>0.05$). The data from volunteer A (Fig. 5.10) showed greater variation, where none of the dentifrices produced significantly different results ($p>0.05$).

5.6.3 Mineral content changes in the Surface Zone and Lesion Body

To investigate if there were any differences in the sites of deposition, a further analysis was carried out on the changes in mineral content of both the surface zone and the lesion body. These are graphically displayed for each volunteer on Figs. 5.19 to 5.21, with a comparison of the significance values detailed in Table 5.4, and can be summarised as follows:

1. For volunteer A (Fig. 19), some of the data began to show some significant differences. As far as an analysis of the surface zone was concerned the non-fluoride was significantly different from the 1000 ppm NaMFP ($p<0.05$) and the 1000 ppm NaMFP dentifrice was significantly different from the 1500 ppm NaMFP dentifrice ($p<0.05$). Significant differences were found also in the lesion body: the non-fluoride product was significantly different from the 1500 ppm NaMFP dentifrice ($p<0.05$), the 1000 ppm from the 1500 ppm ($p<0.05$) and the 1500 ppm from the 2500 ppm dentifrice ($p<0.01$).

2. The analyses of the data from volunteer B (Fig.20) showed the surface zone to be similar to the IML, in that the non-fluoride was significantly different from the 1000 ppm ($p<0.01$), the 1500 ppm ($p<0.01$) and the 2500 ppm ($p<0.05$) NaMFP dentifrices. However, no significant differences were detected between the differing fluoride

concentrations ($p>0.05$). Analyses of the lesion body showed that the only significant differences were between the placebo and the 1000 ppm ($p<0.01$) and the 1500 ppm ($p<0.05$) NaMFP dentifrices. All other comparisons were non-significant ($p>0.05$).

3. Volunteer C (Fig. 21) showed a clear concentration effect in the surface zone. The non-fluoride product was significantly different from all three concentrations of the dentifrices: the 1000 ppm ($p<0.05$), the 1500 ppm ($p<0.01$) and the 2500 ppm ($p<0.01$) NaMFP dentifrices. In addition, volunteer C showed significant differences between the 1000 ppm and the 2500 ppm dentifrices ($P<0.01$) and between the 1500 ppm and the 2500 ppm dentifrices ($p<0.01$). Similar to the analyses of the change in IML, significant differences in the lesion body were found only between the non-fluoride and each of the NaMFP fluoride dentifrices ($p<0.01$), and not between different concentrations of the Fluoride-containing dentifrices ($p>0.05$).

5.7 RESULTS III - THE SODIUM FLUORIDE DENTIFRICES (NaF)

5.7.1 Summary

For the IML data, with volunteers (D & E) significantly increased remineralisation was obtained with the two NaF dentifrices as compared to that of the placebo. No significant differences, however, were detected between the two concentrations of the dentifrice. Further

analyses of the changes in mineral content of the lesion body for volunteer E, however, showed a tendency to a concentration effect. As for the NaMFP dentifrices, a comparison of the significance values for each of the volunteers is detailed in Table 5.5.

5.7.2 Integrated mineral loss changes

In a similar fashion to the results displayed for the NaMFP dentifrices, Figs. 5.22 and 5.23 illustrate the combined remineralisation data for all analysable lesions for each of the volunteers. Figures 5.24 - 5.27 demonstrate examples of the least squares fit to one volunteer, as an example, and the combined data for each of the NaF dentifrice concentrations.

From an analysis of the change in IML, both volunteers using the NaF dentifrices exhibited remineralisation rates superior to the non-fluoride placebo. Volunteer D (Fig. 5.22) showed significant differences between the non-fluoride and both the 1000 ppm and the 1500 ppm NaF dentifrices ($p<0.05$). However, the 1500 ppm NaF appeared to remineralise less well than the 1000 NaF ($p<0.05$). Similarly, volunteer E (Fig. 5.23) remineralised significantly better when using either of the 1000 ppm and 1500 ppm NaF dentifrices, when compared with the non-fluoridated placebo ($p<0.05$). However, no differences were detected between either of the NaF concentrations ($p>0.05$).

5.7.3 Mineral content changes in the Surface Zone and Lesion Body

As before, further analyses were carried out on the changes detected in the surface zone and lesion body for both volunteers. The data are graphically displayed on Figs. 5.28 and 5.29.

1. Volunteer D (Fig. 5.28) showed no differences in the surface zone, between the non-fluoride and the 1000 ppm NaF dentifrices ($p>0.05$), which were both significantly different from the 1500 ppm NaF dentifrice ($p<0.05$). As far as the lesion body was concerned, both the fluoride dentifrices were significantly different from the placebo ($p<0.05$), but in themselves, were not significantly different.

2. Volunteer E (Fig. 5.29) showed no significant differences in the surface zone when comparing either of the two NaF concentrations ($p>0.05$). However, each was significantly different from the placebo ($p<0.05$). A greater difference and possible concentration effect, however, was detected in the mineral content change of the lesion body. The 1000 ppm NaF was significantly different from the placebo ($p<0.01$), which in turn was bordering on significance when compared to the 1500 ppm NaF dentifrice ($p = 0.05$).

5.8 DISCUSSION

The aim of this part of the project, as stated previously, was to investigate the effect of dentifrice fluoride concentration, from NaMFP and NaF, on the remineralisation of artificial caries-like lesions in enamel, using the *in situ* appliance developed in Chapter 4. It would appear to have linked successfully the advantages of the single section technique and microradiography/ microdensitometry, and was a useful method of assessing mineral deposition within the artificial white-spot lesion. Remineralisation, as discussed in detail in Chapter 1, is now known to be the major cariostatic effect of topically applied fluorides. Fluoride increases the rate of repair of carious enamel lesions, and is preferentially taken up by porous enamel rather than normal unaltered enamel. The results from this Chapter have shown conclusively that for the majority of subjects, the fluoridated dentifrices (of both NaMFP and NaF types) have exhibited a greater remineralising potential than either of the placebo dentifrices. This observation is in agreement with the *in vivo*/*in situ* work of Featherstone *et al*. (1982) and Mellberg *et al*. (1985) who have reported significant differences in remineralising ability between test and control dentifrices.

As far as the apparent inability to show a relationship between fluoride concentration and the amount of mineral redeposited within the lesion, is interesting and worthy

of further comment. From a chemical standpoint, this is not surprising as it has been shown previously that the effect of fluoride on the acid dissolution rate of enamel is related to the logarithm of fluoride concentration (Forward, 1980). Thus, increasing oral fluoride concentrations from 1000 ppm to 2500 ppm will not increase fluoride activity to any marked extent. Forward (1980) concluded that increasing the fluoride concentration will not be a practical method of substantially improving the effectiveness of a fluoride dentifrice.

In a large scale clinical trial using the same three NaMFP dentifrices employed in this study (Stephen et al., 1987), a relationship between dentifrice fluoride concentration and enhancement of caries protection has been reported. The results of that trial are in agreement with many other epidemiological studies (Hodge et al., 1980; Buhe et al., 1984), who have reported a dose-related enhancement of caries protection when comparing a range of dentifrice fluoride concentrations of between zero and 1500 ppm. However, other workers have failed to show any such association (Forsman, 1974; Koch et al., 1982; Mainwaring & Naylor, 1983; Juliano et al., 1985; Ripa et al., 1987). In addition, Reintsema et al. (1985), from in vivo/in situ investigations, have been unable to demonstrate a concentration effect. With regard to the concentration of fluoride tested, only in recent years have clinical trials tested dentifrice fluoride concentrations of up to 2500 ppm, and have employed a positive control dentifrice of 1000 ppm (Ripa et al., 1987; Stephen et al., 1987).

Stephen _et al_. (1987) have shown an increase in caries protection when increasing the dentifrice fluoride concentration from 1000 ppm to 2500 ppm, whilst Ripa _et al_. (1987) reported no such association. In the latter study, however, a poor compliance rate was noted, the remainder either failing to use the dentifrice at all, or choosing an alternative marketed paste. Thus, the stated conclusions from that trial are drawn from data which have a marked influence of a majority of noncompliant subjects, and therefore, should not exclude the possibility of a concentration effect. Until the study of Stephen _et al_. (1987), the clinical trials supporting an increased benefit from higher fluoride concentration have employed dentifrice fluoride concentrations from 250 ppm to 1000 ppm. In those trials, carried out less recently (when the rates of carious attack were probably higher), low levels of dentifice fluoride (250 ppm etc.) were likely to show very little difference in caries protection when compared with a nonfluoridated control, but would still be inferior to the 1000 ppm (de Kloet _et al_., 1987). Thus, a concentration effect may have been more easlily exhibited in those earlier years, when diet may have been more cariogenic and alternative fluoridated dentifrices were less readily available.

The further analyses of the changes in mineral content in the surface zone and lesion body was a useful exercise, and worthy of further comment. Whilst an analysis of total mineral gain or loss (IML) is known to be more sensitive to changes in mineral content within the lesion

(Strang _et al_., 1986), site specific analyses do have an important role. This view is further substantiated by the further analyses from volunteers C and E, both of whom showed a demonstrable association between fluoride concentration and mineral deposition within the lesion. This was apparent in the surface zone for volunteer C, and in the lesion body for volunteer E. Further work is necessary, employing a greater number of subjects to substantiate this finding.

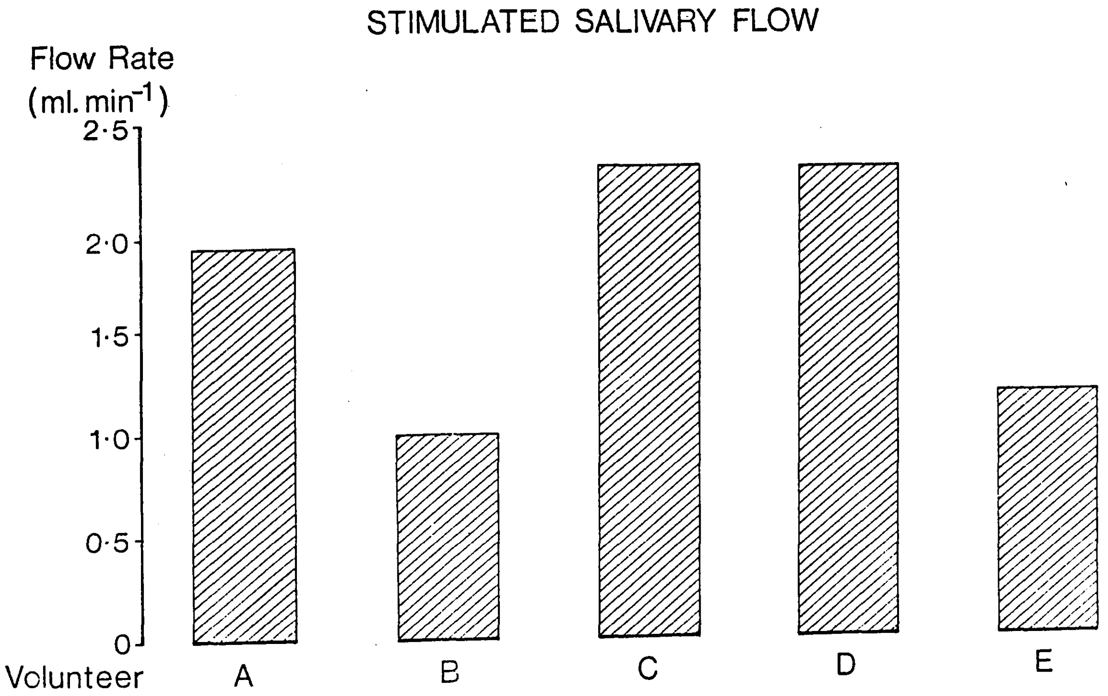

Figure 5.1 Plot of stimulated salivary flow for each of the five volunteers (A - E).

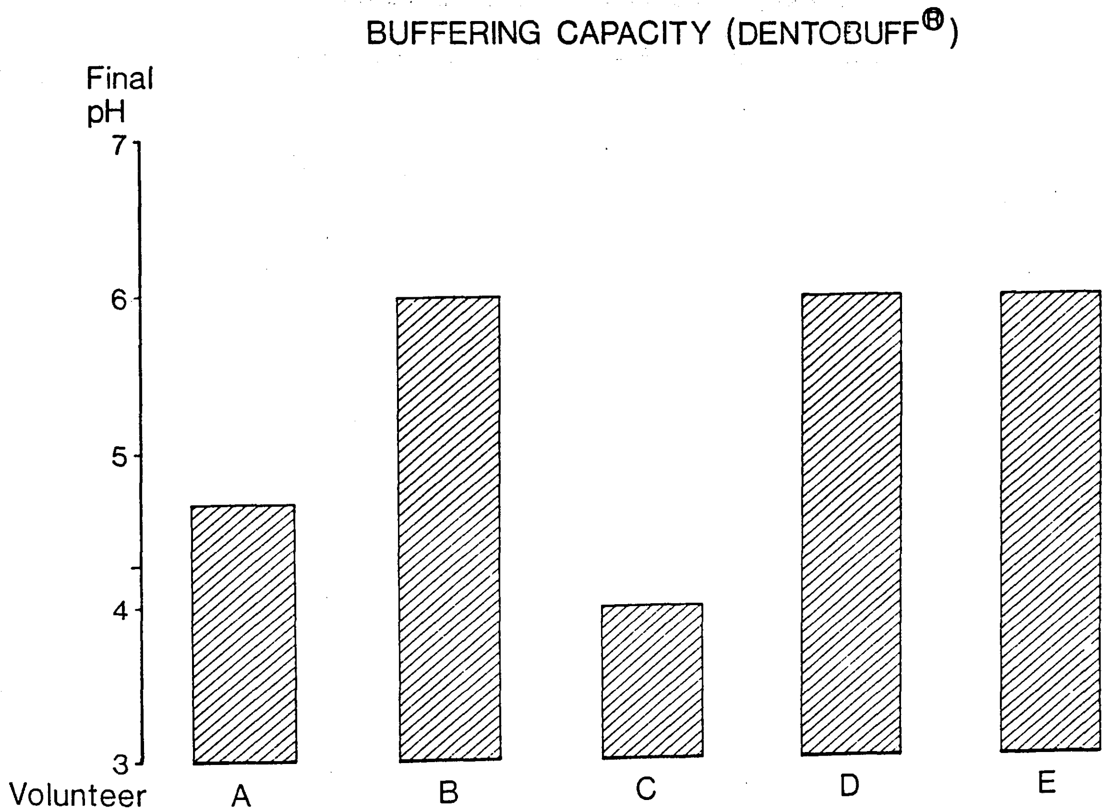

Figure 5.2 Plot of the measured buffering capacity for each of the five volunteers (A - E) using the Dentobuff kit.

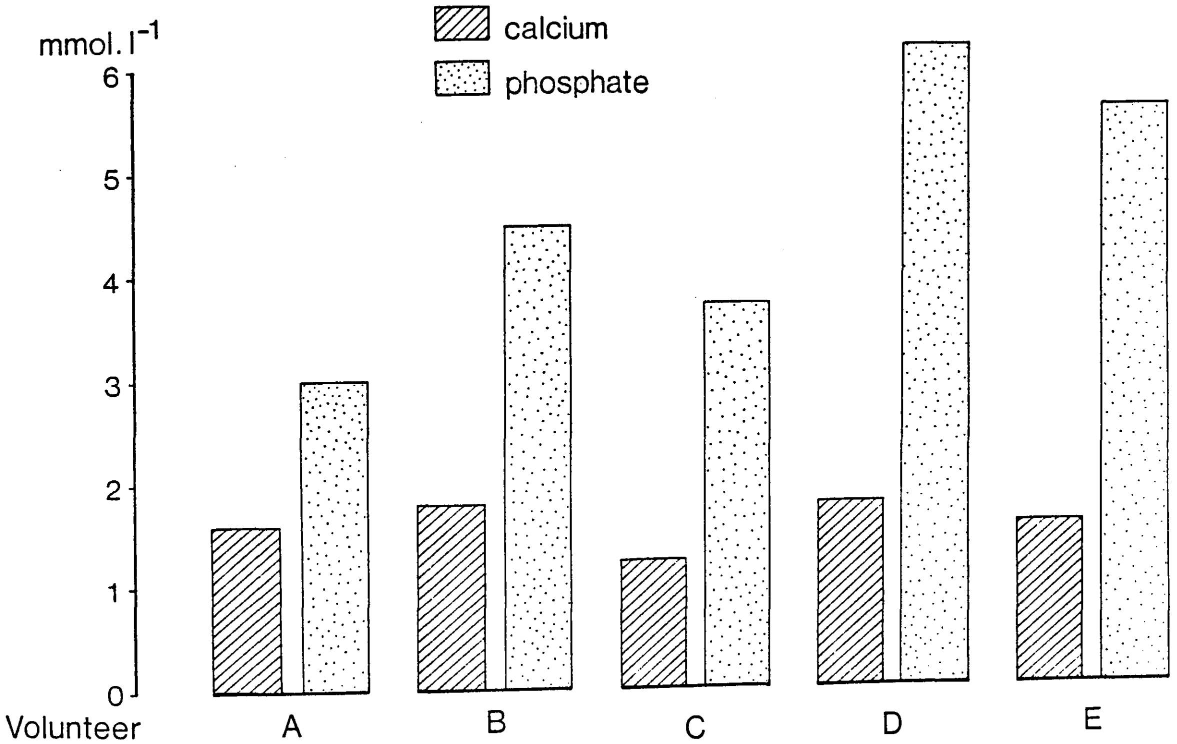

Figure 5.3 Plot of the stimulated salivary calcium and phosphate levels for each of the five volunteers (A - E).

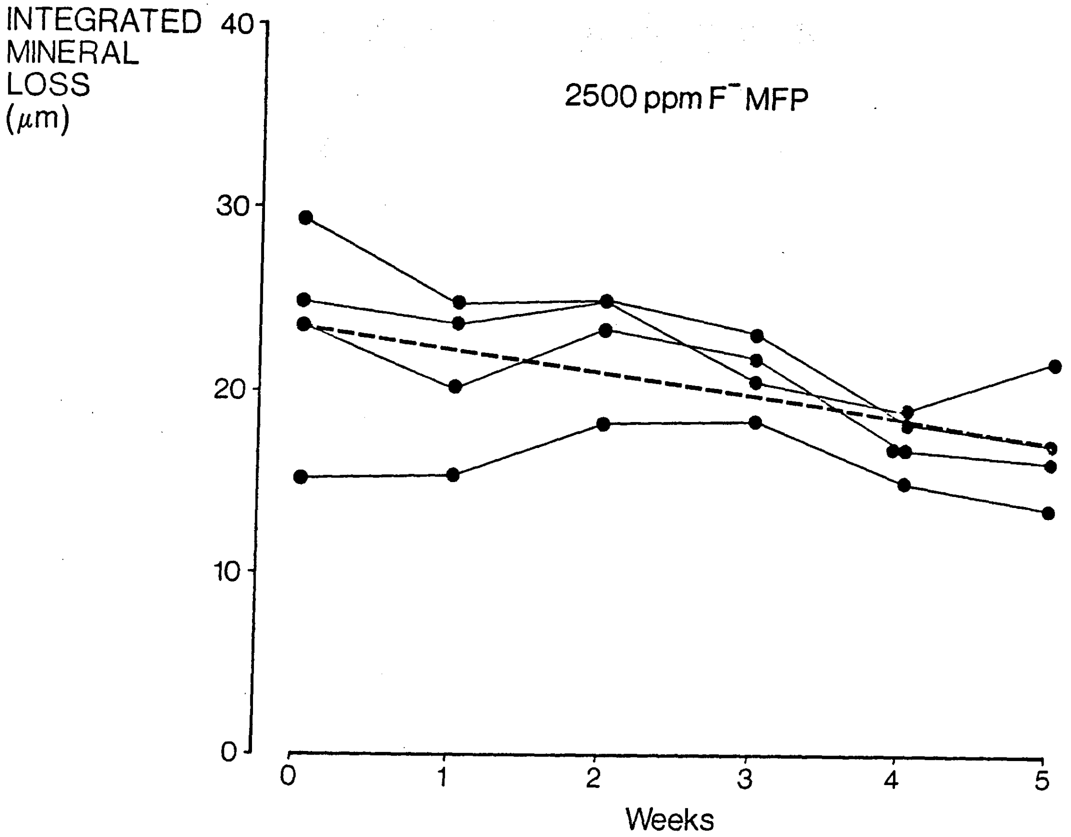

Figure 5.4 Example of the "least squares fit" (dotted line) to the change in integrated mineral loss (IML) for four lesions exposed to the 2500 ppmF NaMFP dentifrice.

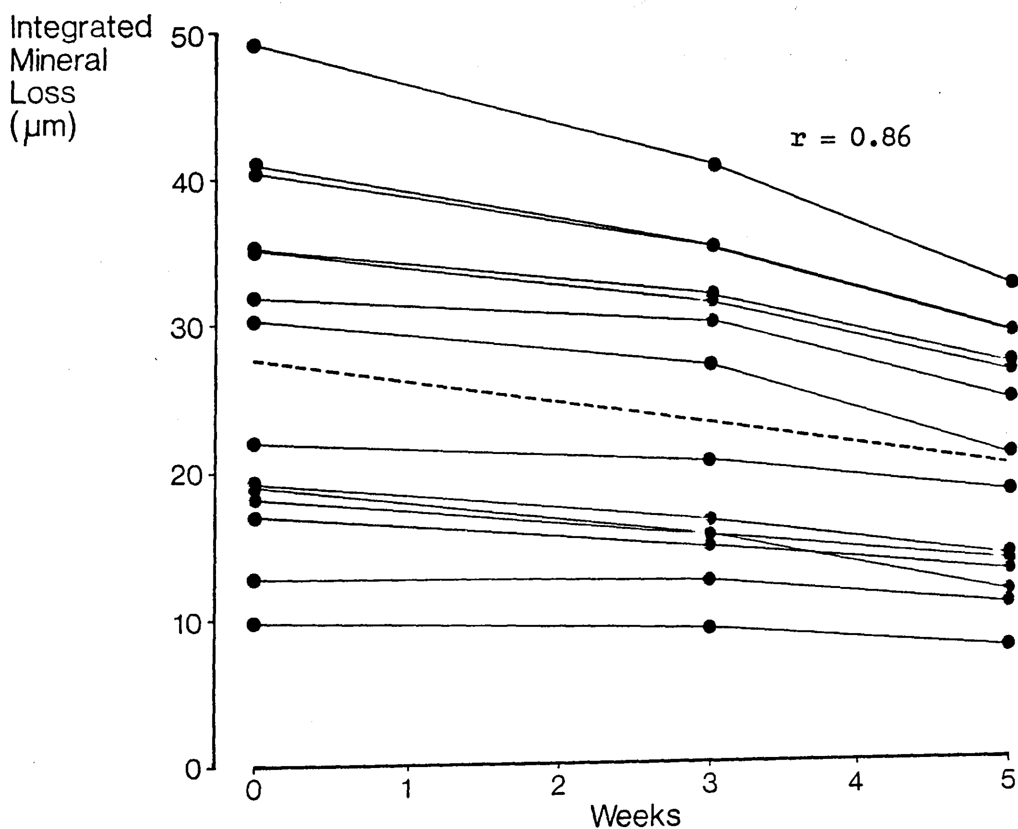

Figure 5.5 Example of the "least squares fit" (dotted line) to the change in integrated mineral loss (IML) for all selected lesions of volunteer B (1000 ppmF NaMFP dentifrice).

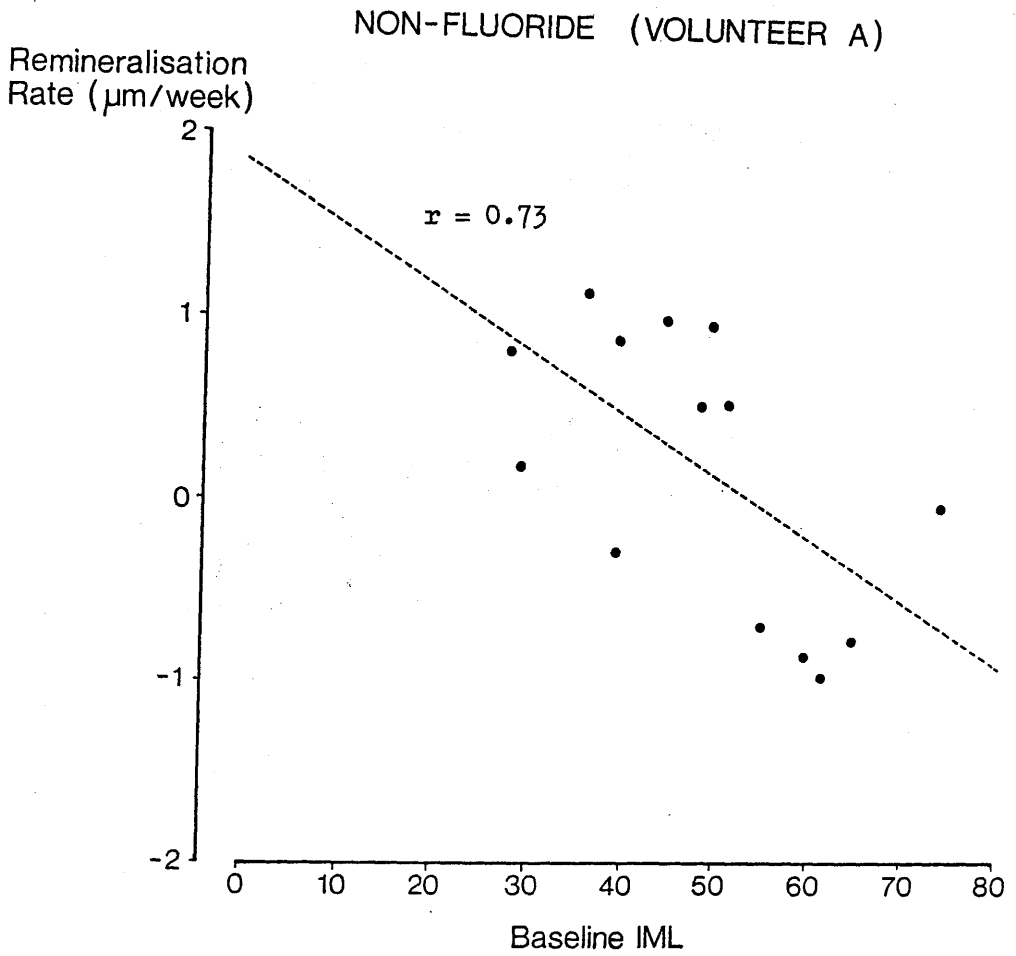

Figure 5.6 Plot of remineralisation rate (change in IML) against initial lesion size (IML) for volunteer A, when using the non-F placebo dentifrice.

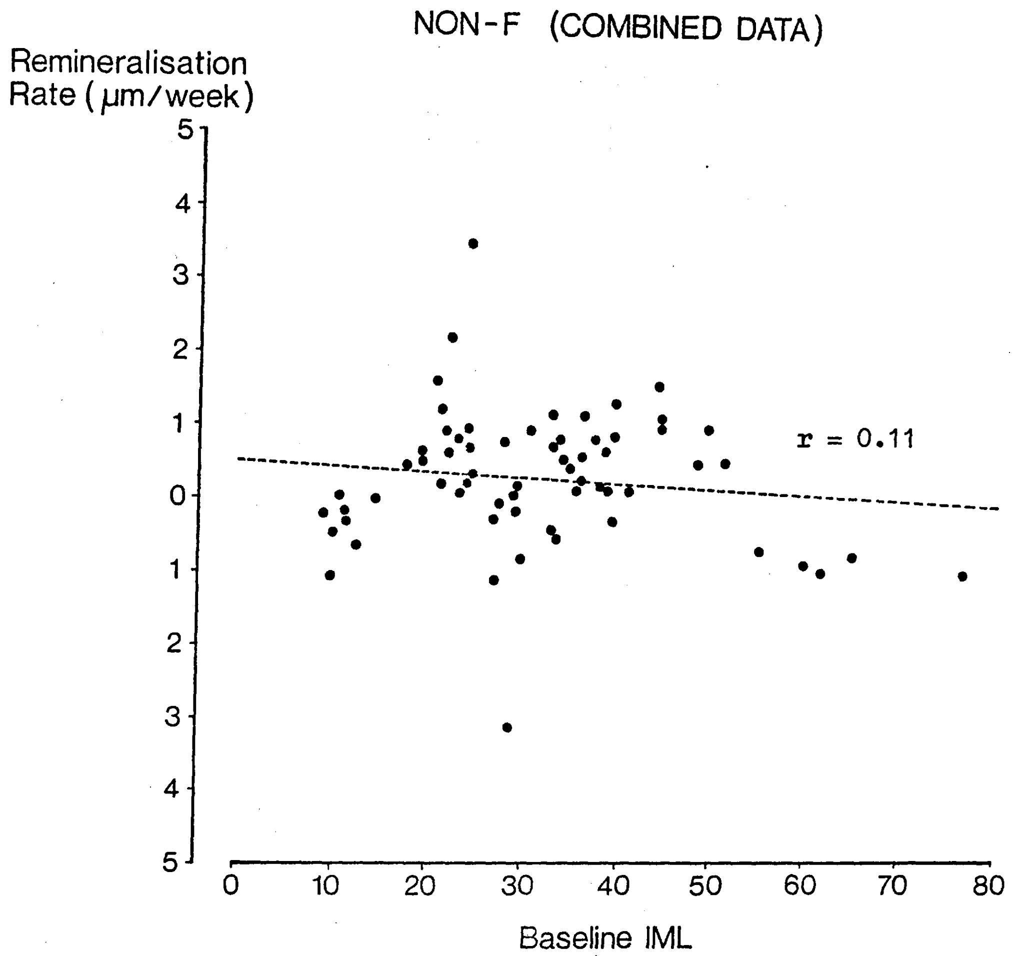

Figure 5.7 Plot of remineralisation rate (change in IML) against initial lesion size (IML) for all five volunteers when using the placebo dentifrice.

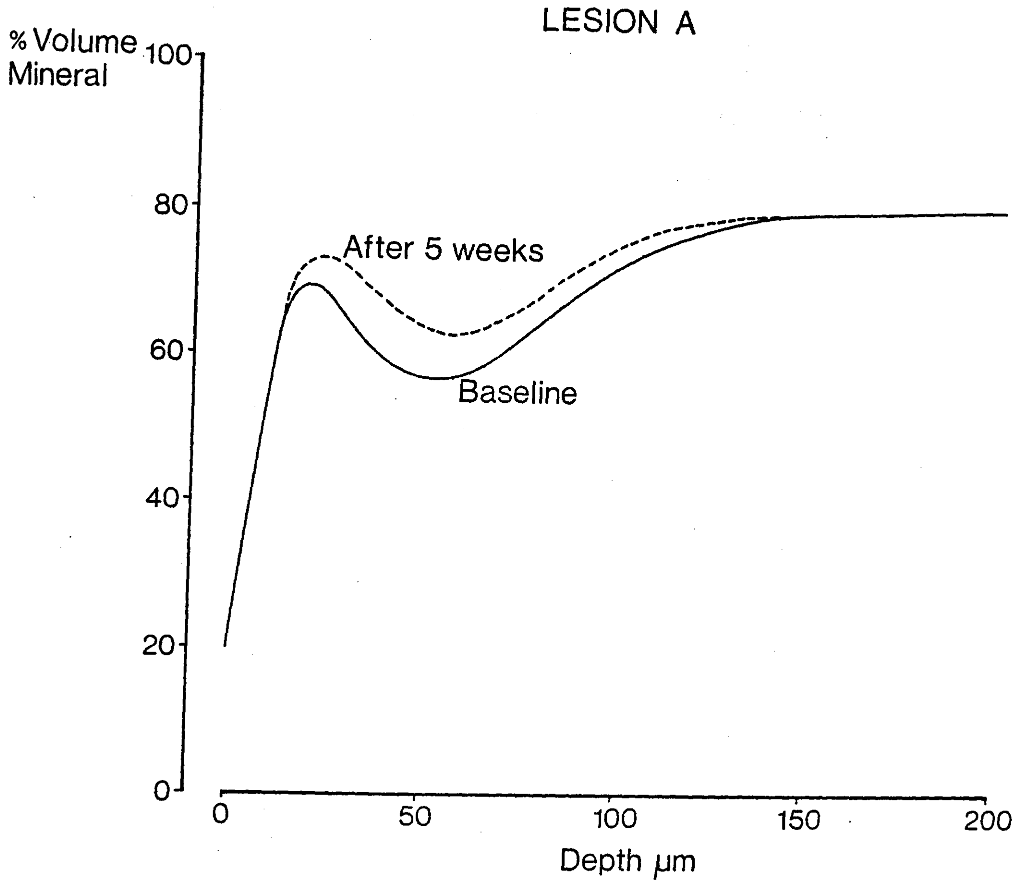

Figure 5.8 Typical microdensitometric scans for 1 lesion at baseline and after 5 weeks' intraoral exposure to the 2500 ppmF NaMFP dentifrice (volunteer C).

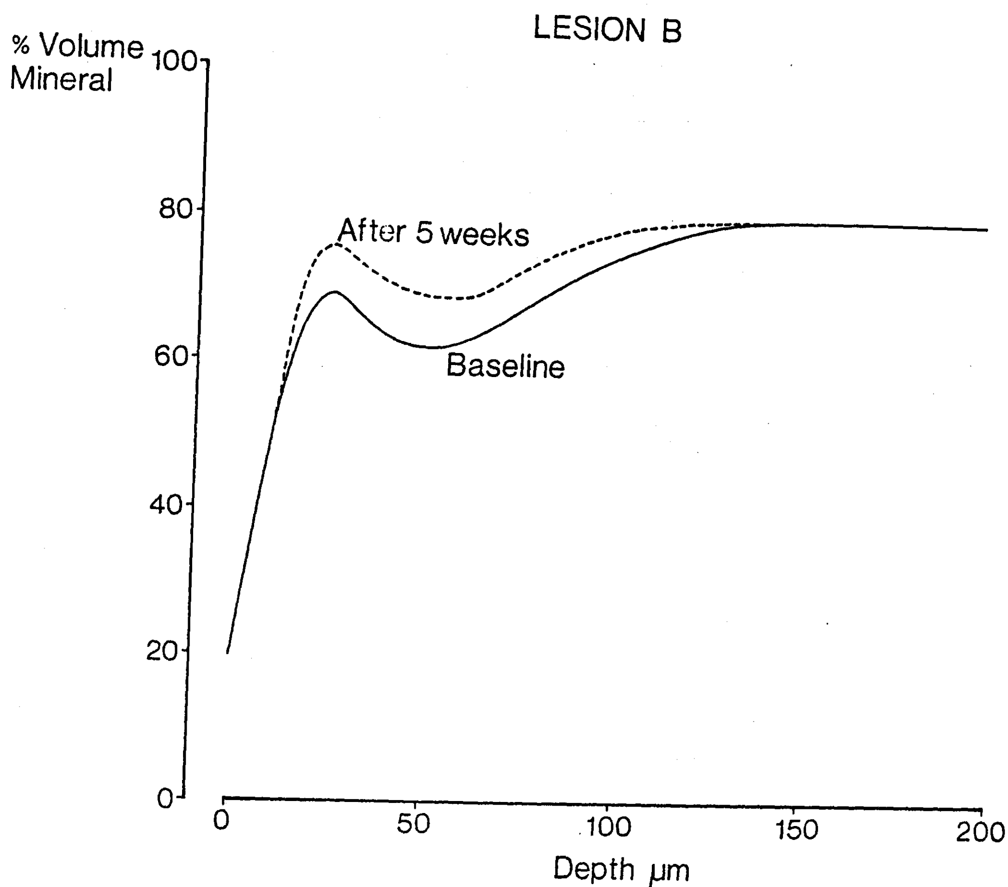

Figure 5.9 Typical microdensitometric scans for 1 lesion at baseline and after 5 weeks' intraoral exposure to the 1000 ppmF NaMFP dentifrice (volunteer B).

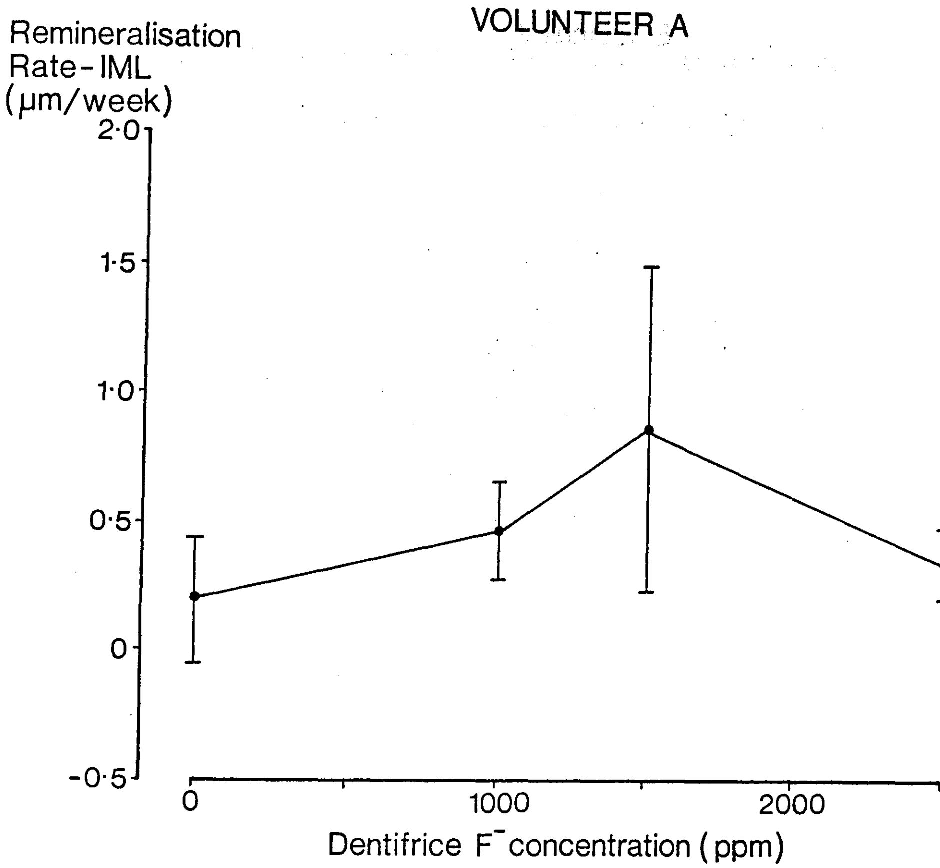

Figure 5.10 Plot of remineralisation rate against NaMFP dentifrice concentration for volunteer A (error bars ± 1 S.E.).

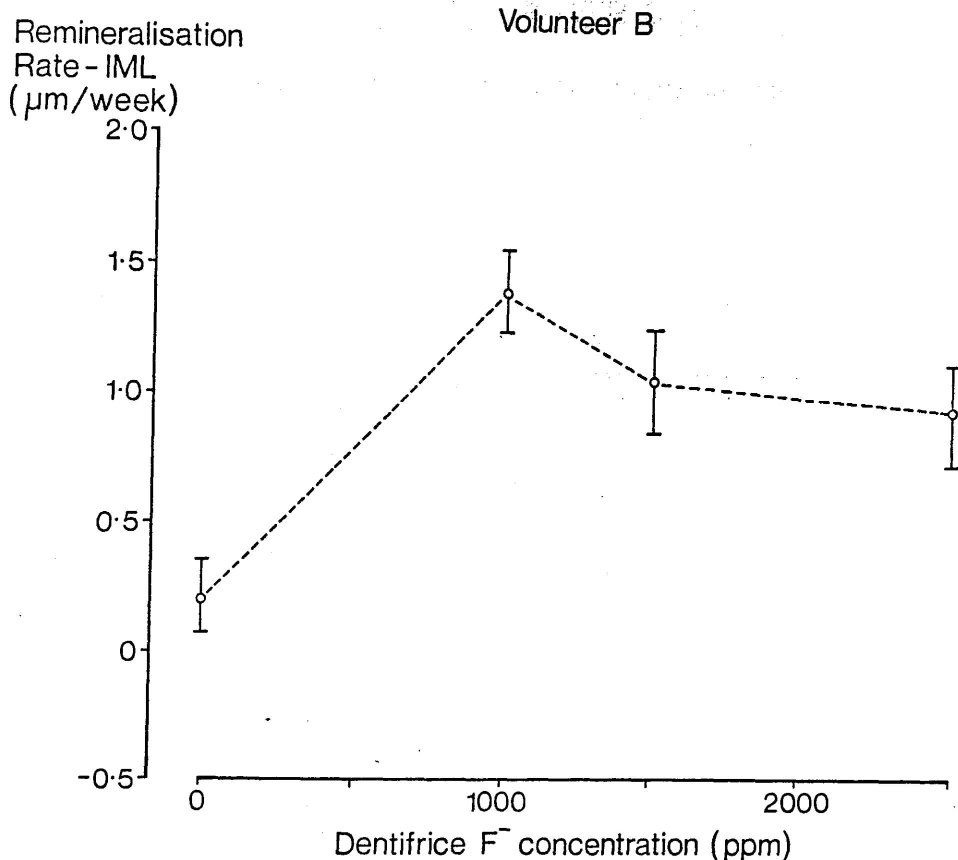

Figure 5.11 Plot of remineralisation rate against NaMFP dentifrice concentration for volunteer B (error bars ± 1 S.E.).

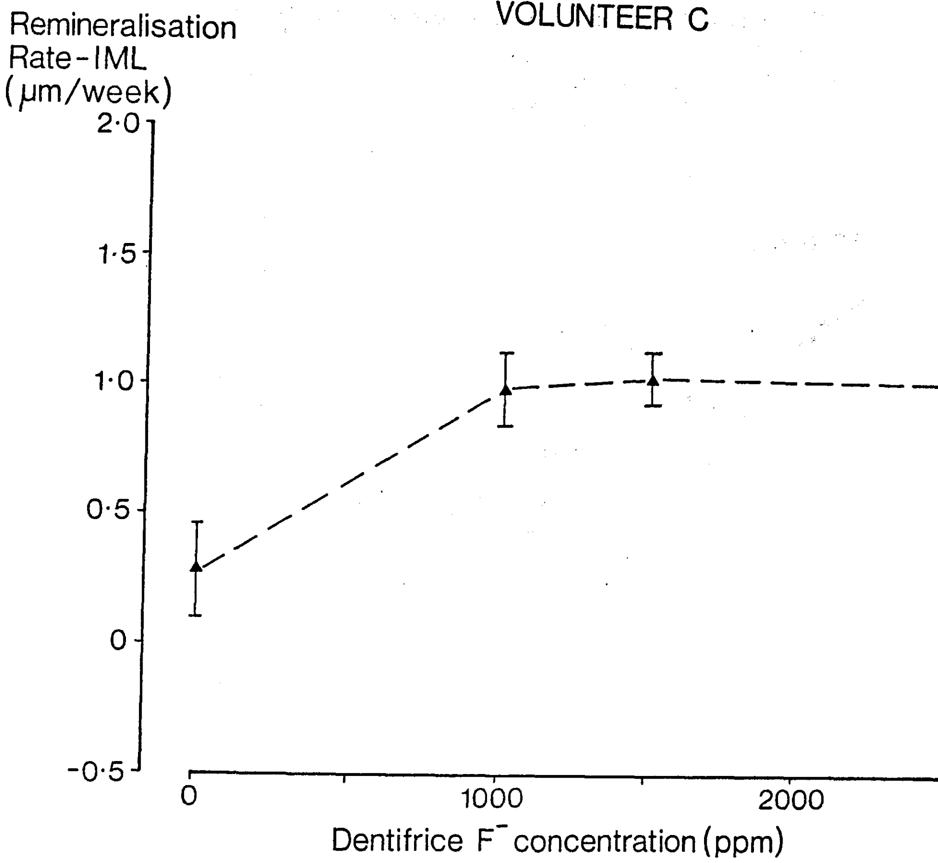

Figure 5.12 Plot of remineralisation rate against NaMFP dentifrice concentration for volunteer C (error bars ± 1 S.E.).

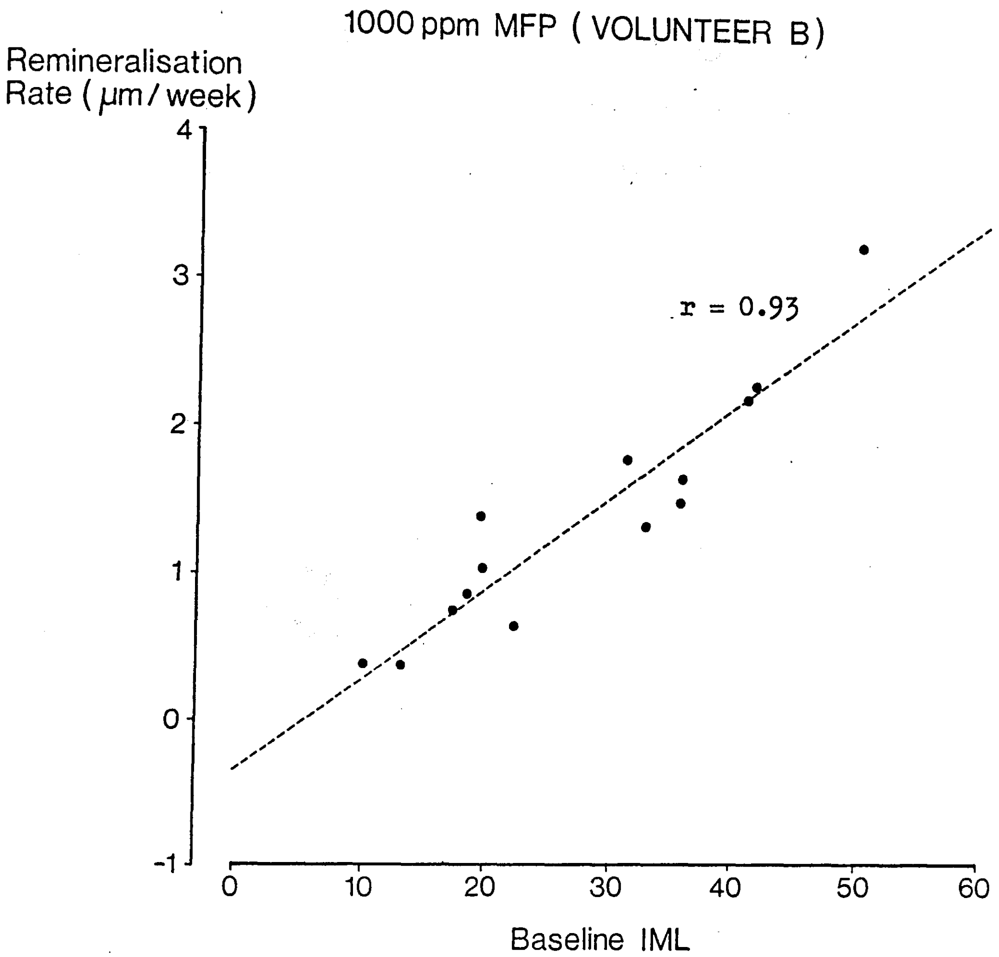

Figure 5.13 Plot of remineralisation rate against initial lesion size (IML) for volunteer B when using the 1000 ppmF NaMFP dentifrice.

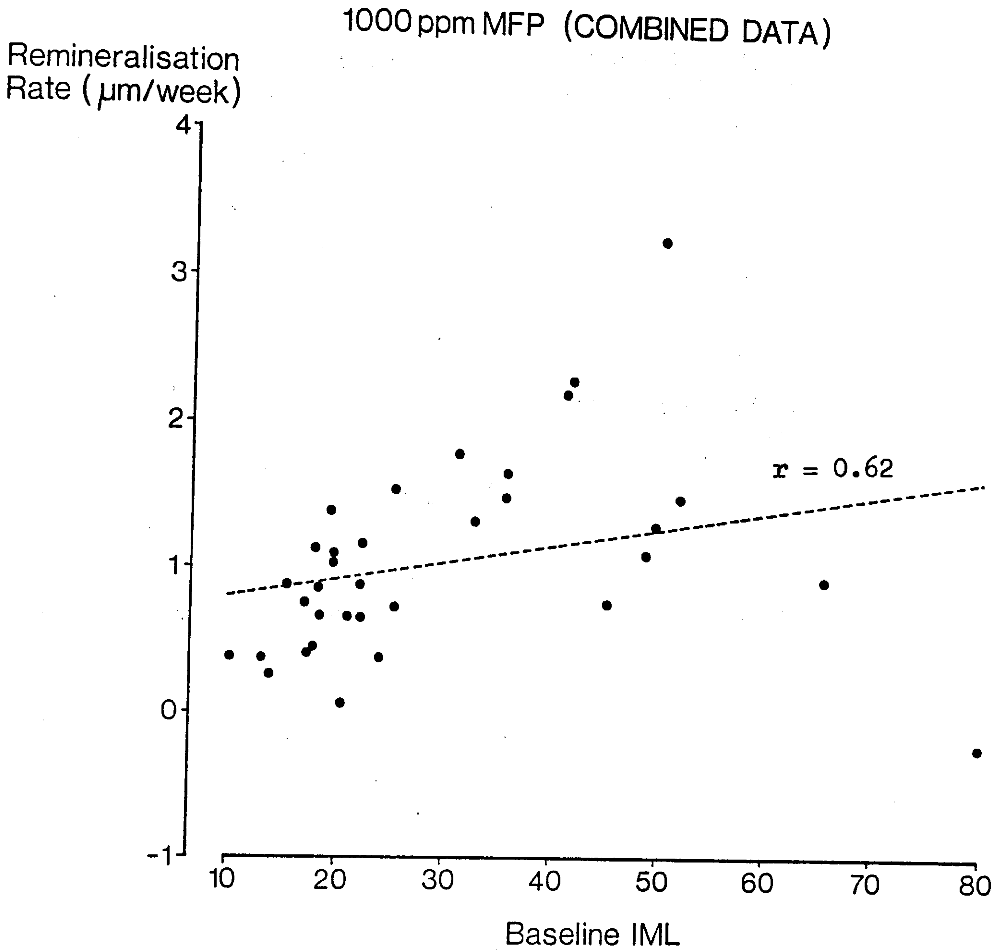

Figure 5.14 Plot of remineralisation rate against initial lesion size (IML) for all volunteers when using the 1000 ppmF NaMFP dentifrice.

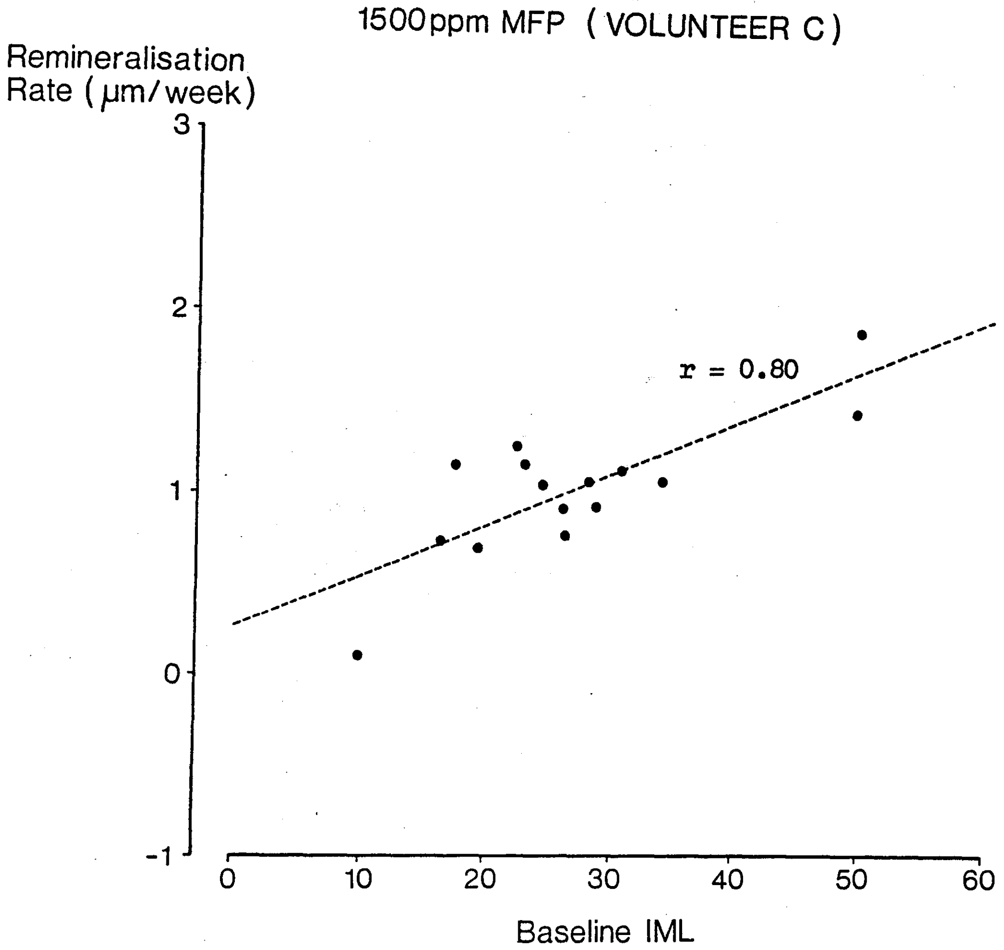

Figure 5.15 Plot of remineralisation rate against initial lesion size (IML) for volunteer C when using the 1500 ppmF NaMFP dentifrice.

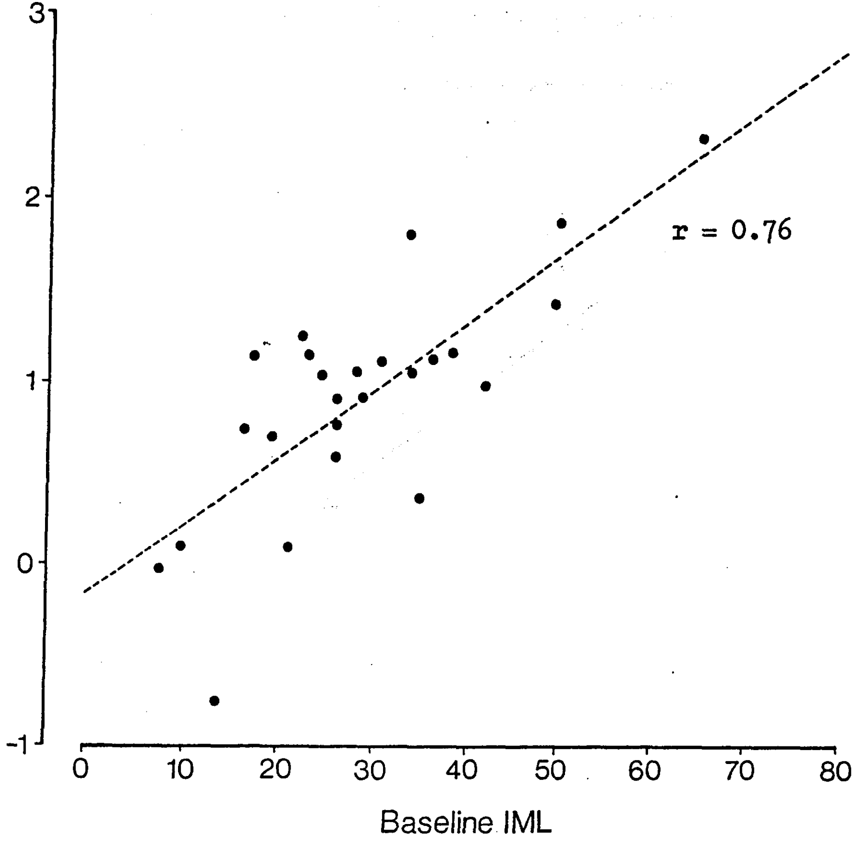

Figure 5.16 Plot of remineralisation rate against initial lesion size (IML) for all volunteers when using the 1500 ppmF NaMFP dentifrice.

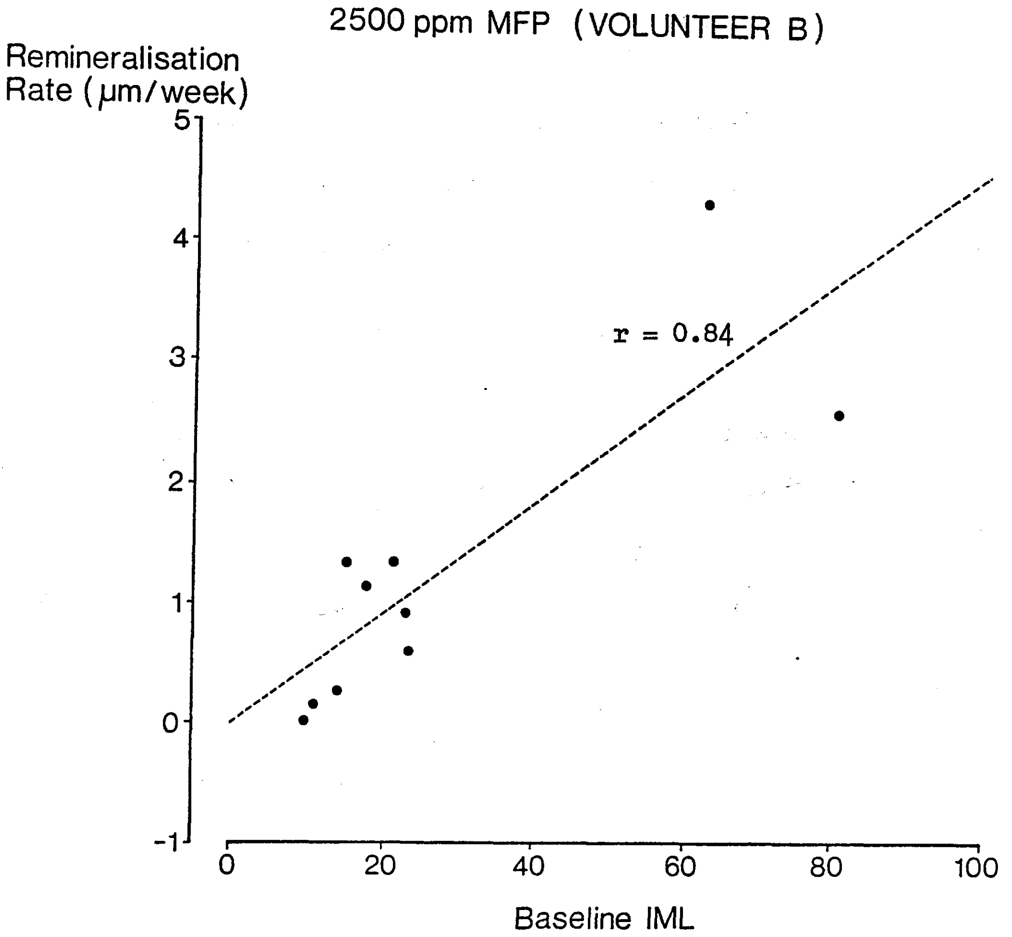

Figure 5.17 Plot of remineralisation rate against initial lesion size (IML) for volunteer B when using the 2500 ppmF NaMFP dentifrice.

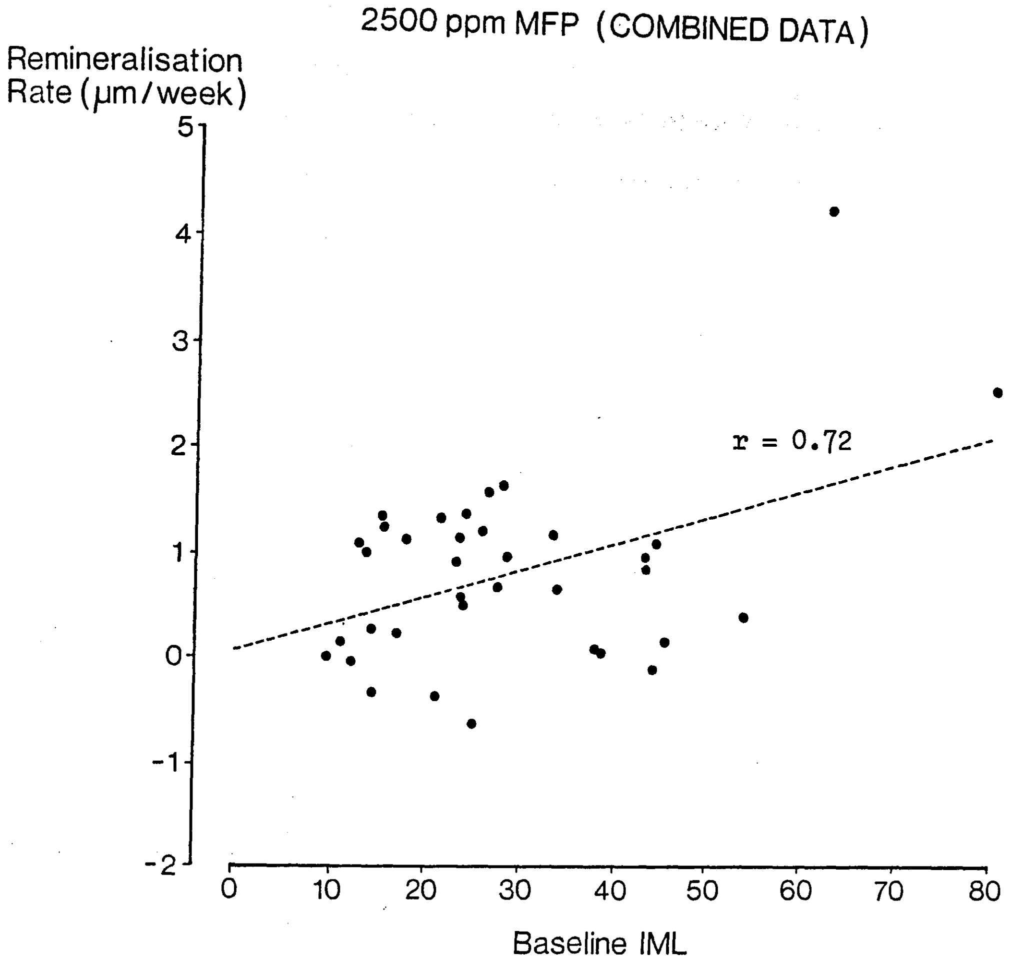

Figure 5.18 Plot of remineralisation rate against initial lesion size (IML) for all volunteers when using the 2500 ppmF NaMFP dentifrice.

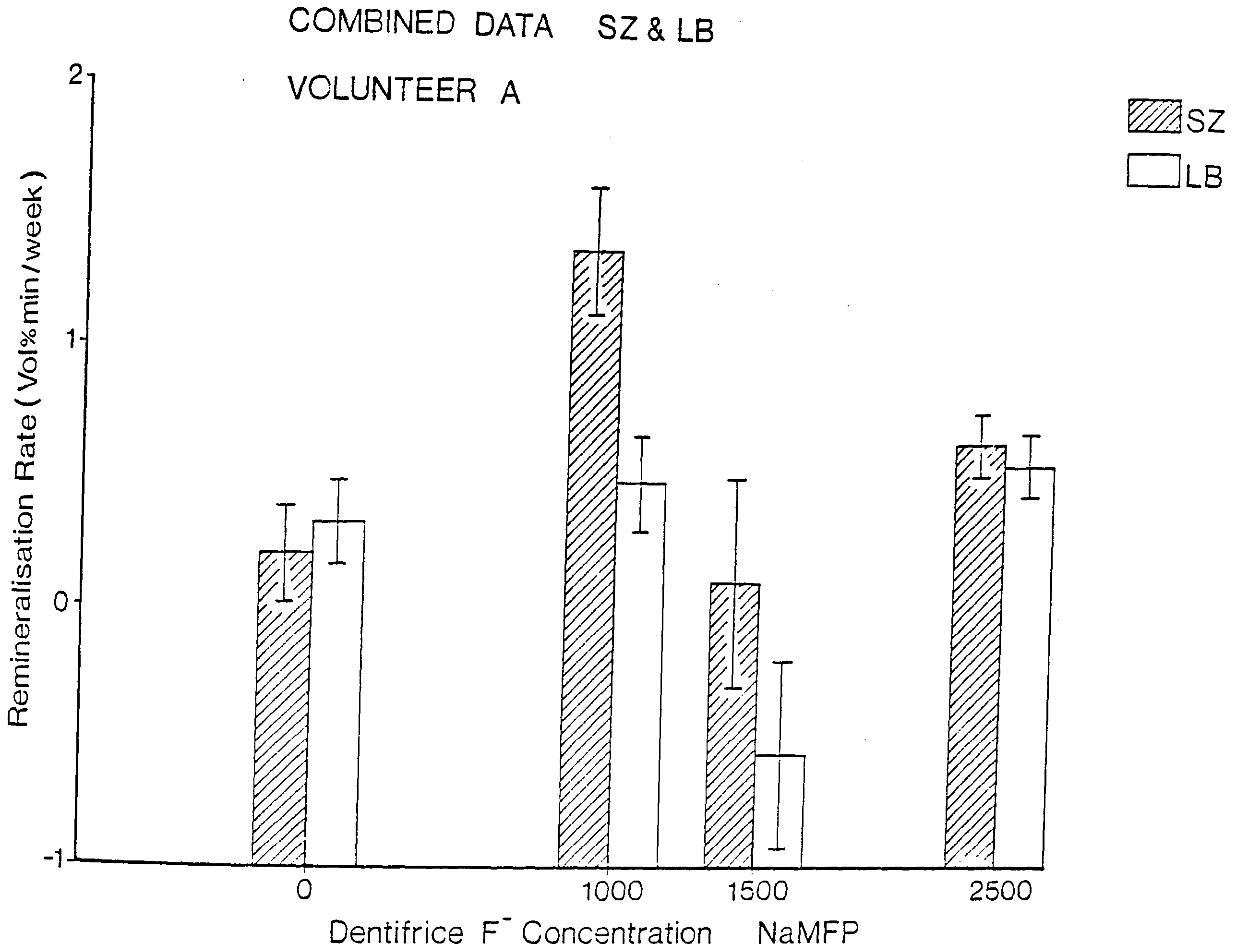

Figure 5.19 Plot of surface zone (SZ) and lesion body (LB) remineralisation rates (% volume mineral/week) against NaMFP dentifrice concentration for volunteer A. (error bars ± 1 S.E.).

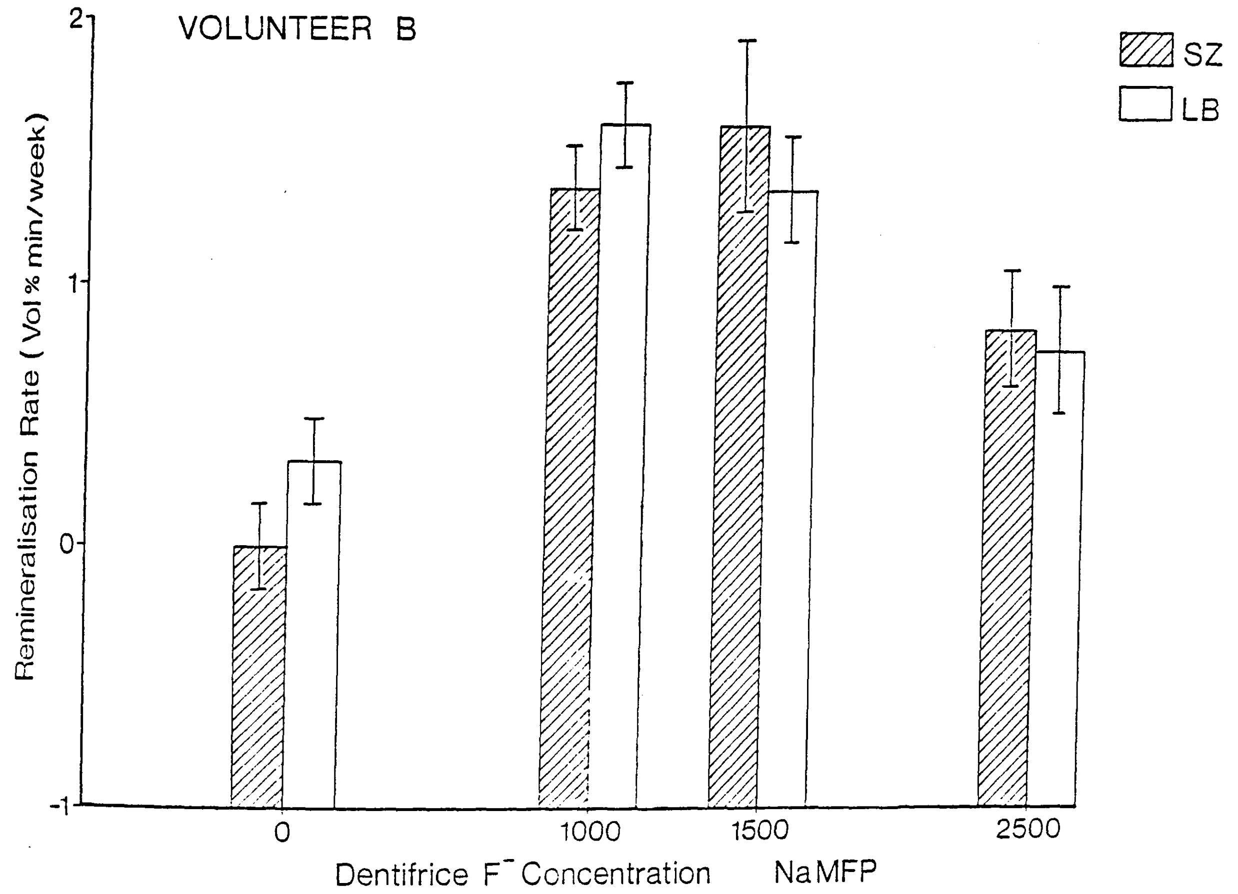

Figure 5.20 Plot of surface zone (SZ) and lesion body (LB) remineralisation rates (% volume mineral/week) against NaMFP dentifrice concentration for volunteer B. (error bars ± 1 S.E.).

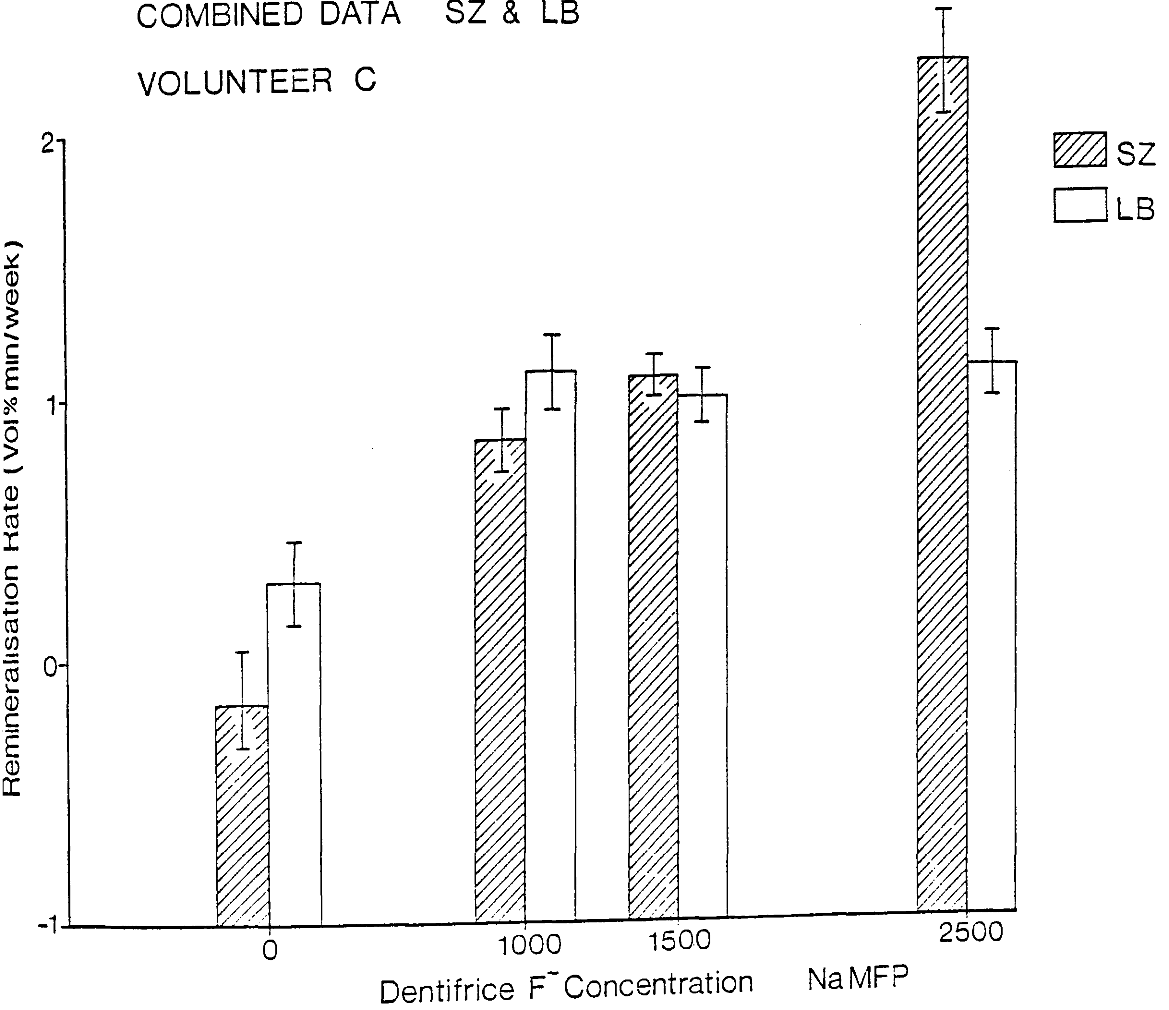

Figure 5.21 Plot of surface zone (SZ) and lesion body (LB) remineralisation rates (% volume mineral/week) against NaMFP dentifrice concentration for volunteer C. (error bars ± 1 S.E.).

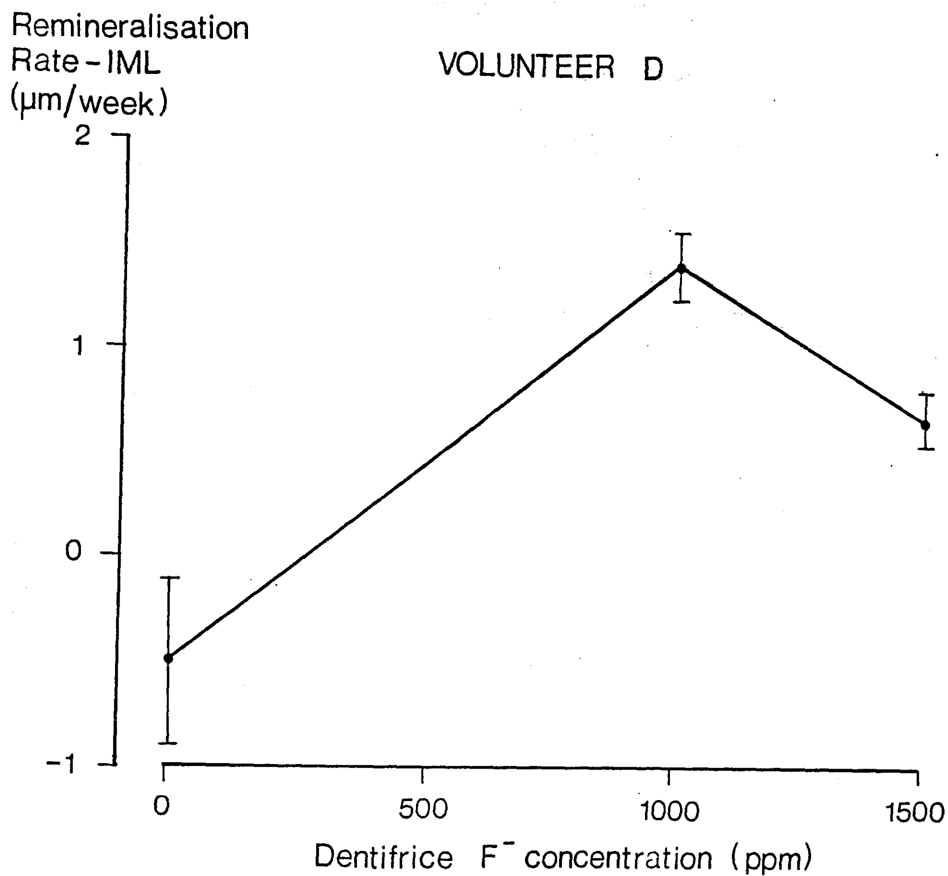

Figure 5.22 Plot of remineralisation rate against NaF dentifrice concentration for volunteer D (error bars ± 1 S.E.).

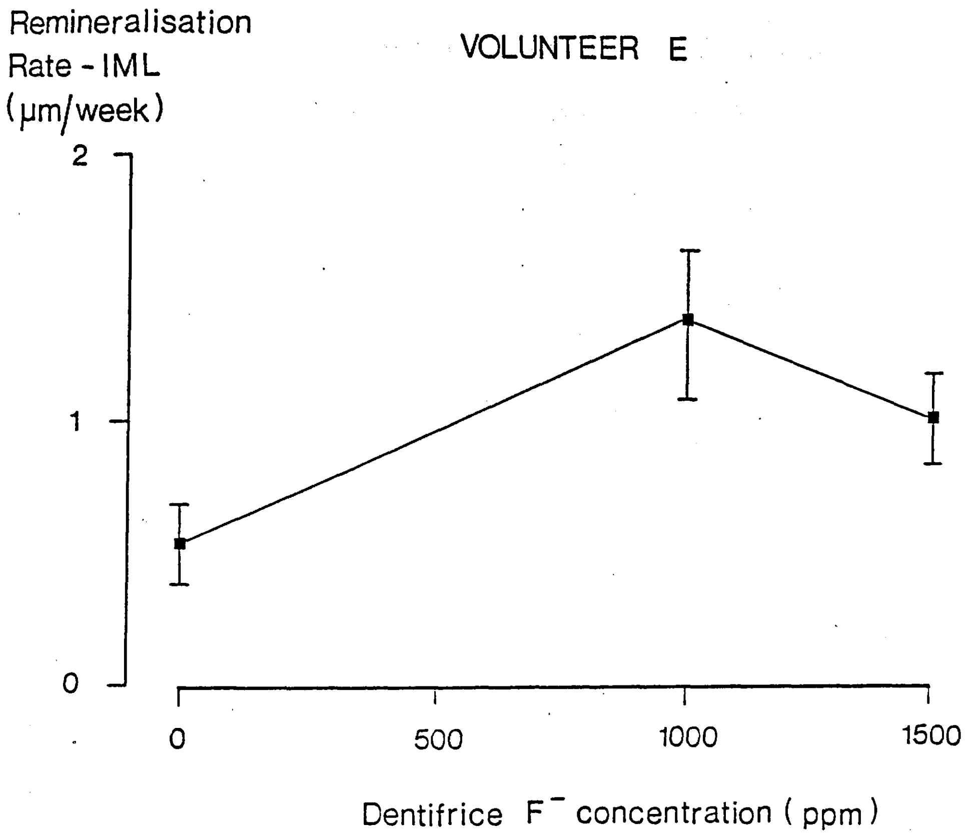

Figure 5.23 Plot of remineralisation rate against NaF dentifrice concentration for volunteer E (error bars $\pm$ 1 S.E.).

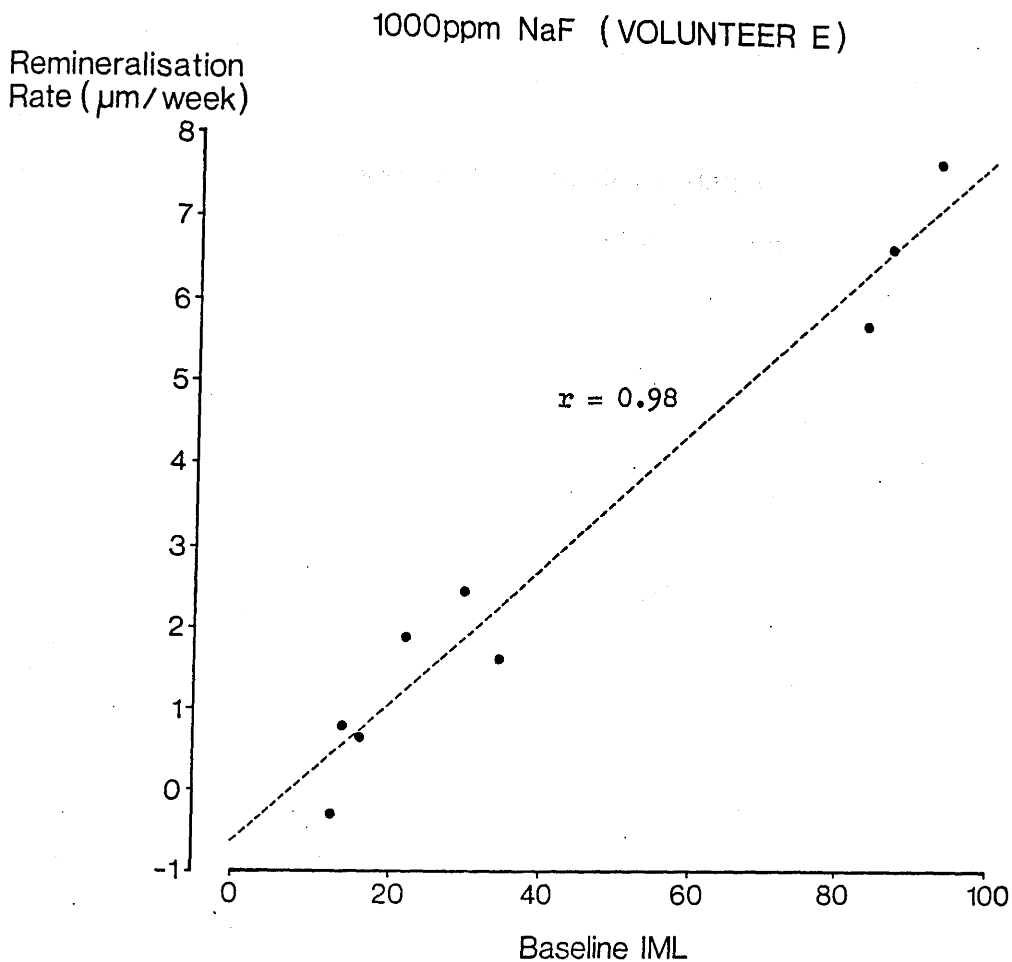

Figure 5.24 Plot of remineralisation rate against initial lesion size (IML) for volunteer when using the 1000 ppmF NaF dentifrice.

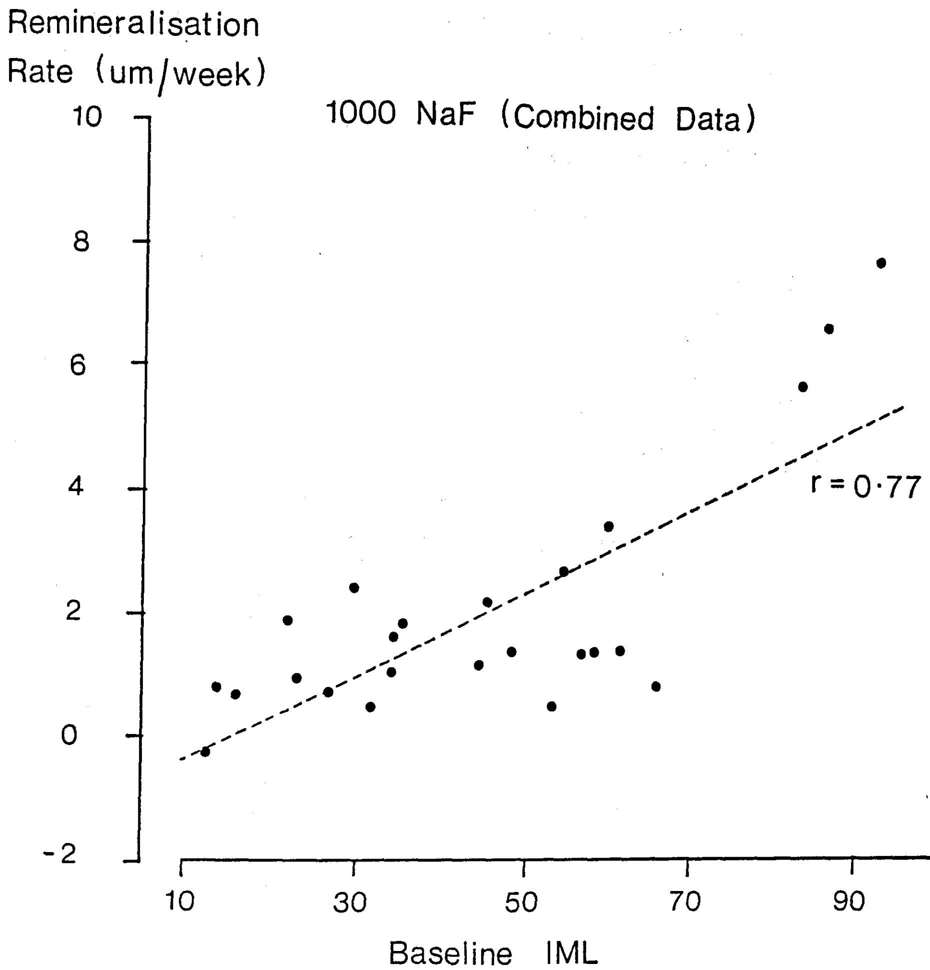

Figure 5.25 Plot of remineralisation rate against initial lesion size (IML) for both volunteers when using the 1000 ppmF NaF dentifrice.

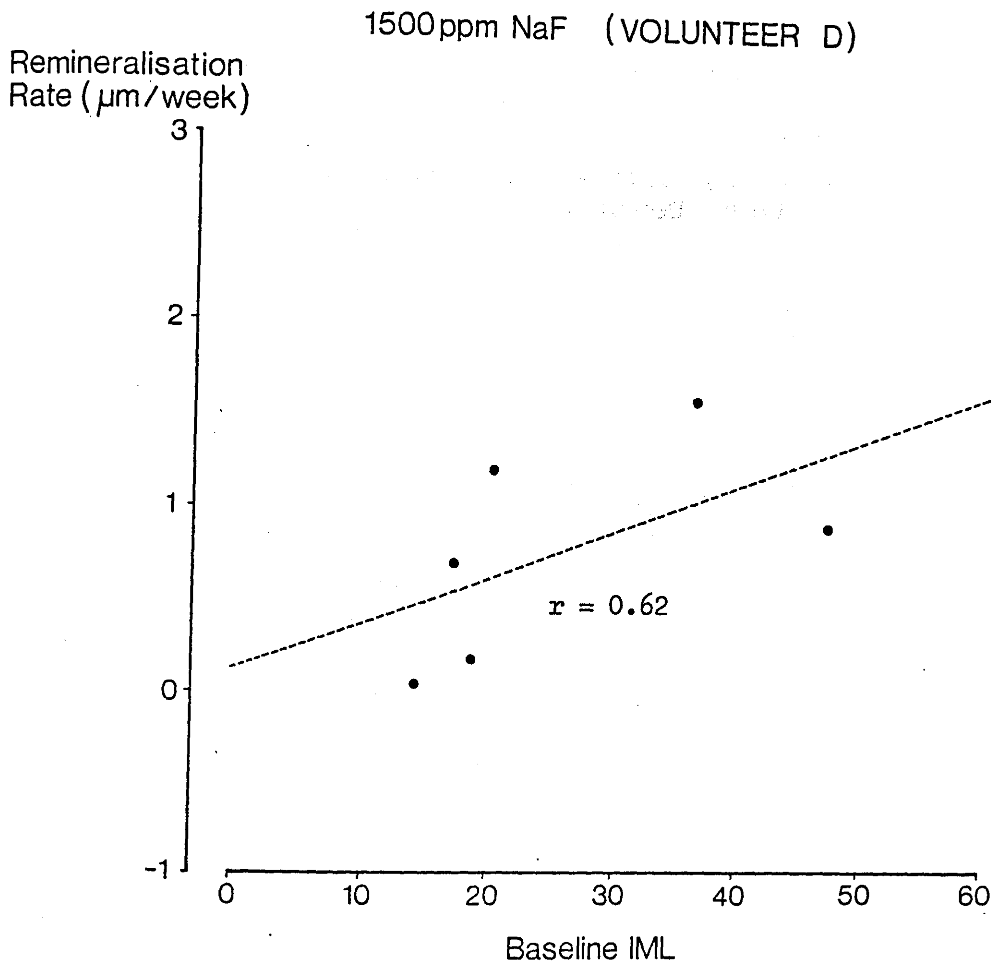

Figure 5.26 Plot of remineralisation rate against initial lesion size (IML) for volunteer D when using the 1500 ppmF NaF dentifrice.

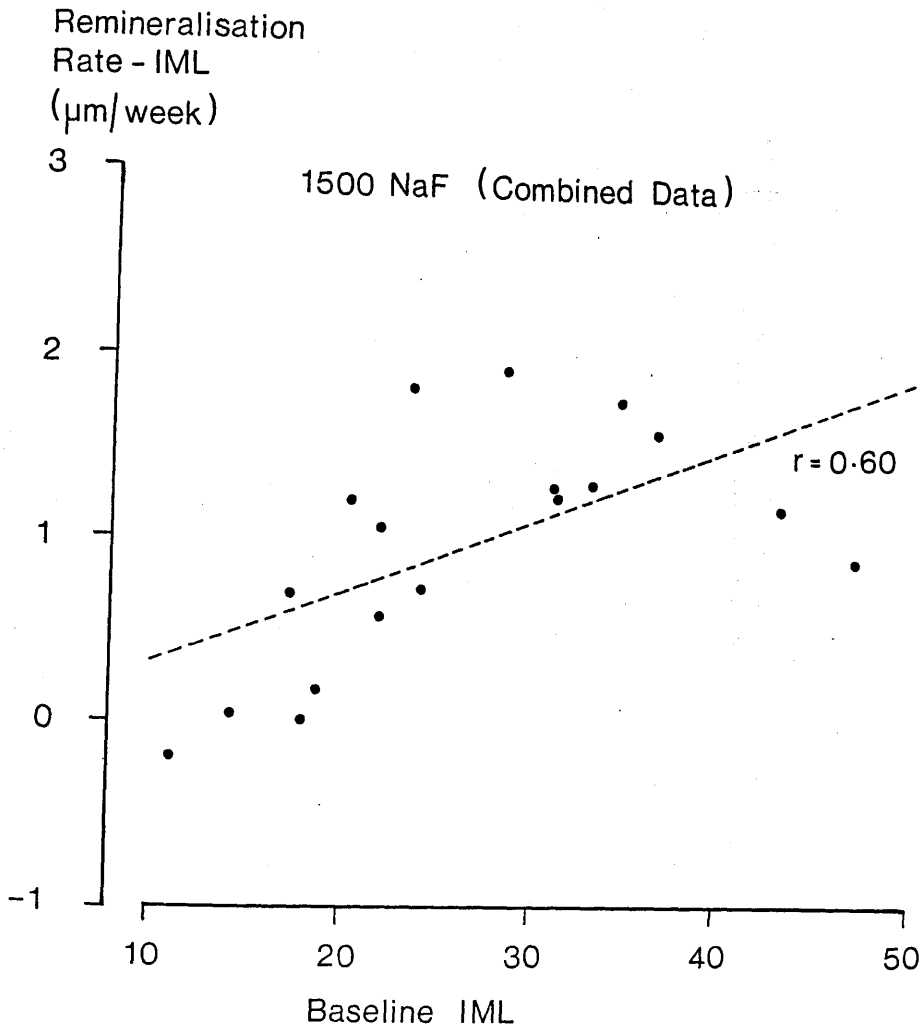

Figure 5.27 Plot of remineralisation rate against initial lesion size (IML) for both volunteers when using the 1500 ppmF NaF dentifrice.

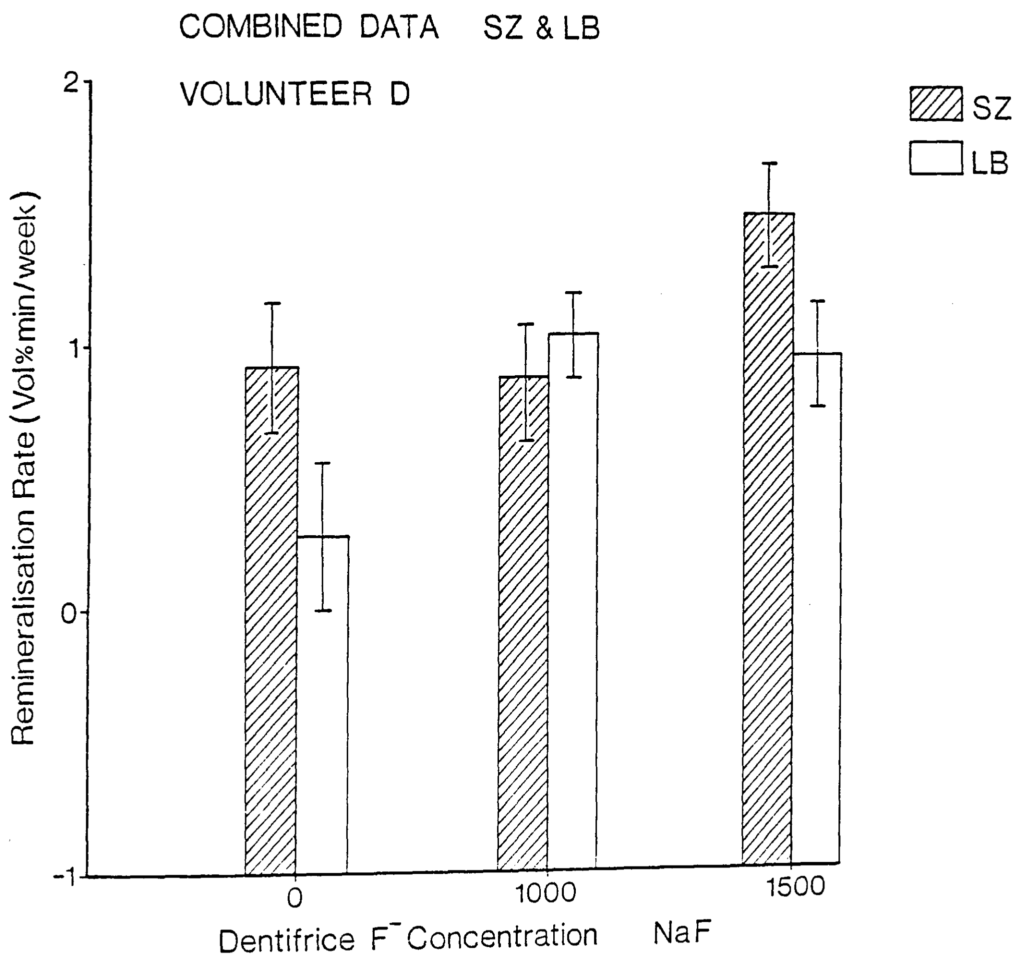

Figure 5.28 Plot of surface zone (SZ) and lesion body (LB) remineralisation rates (% volume mineral/week) against NaF dentifrice concentration for volunteer D.

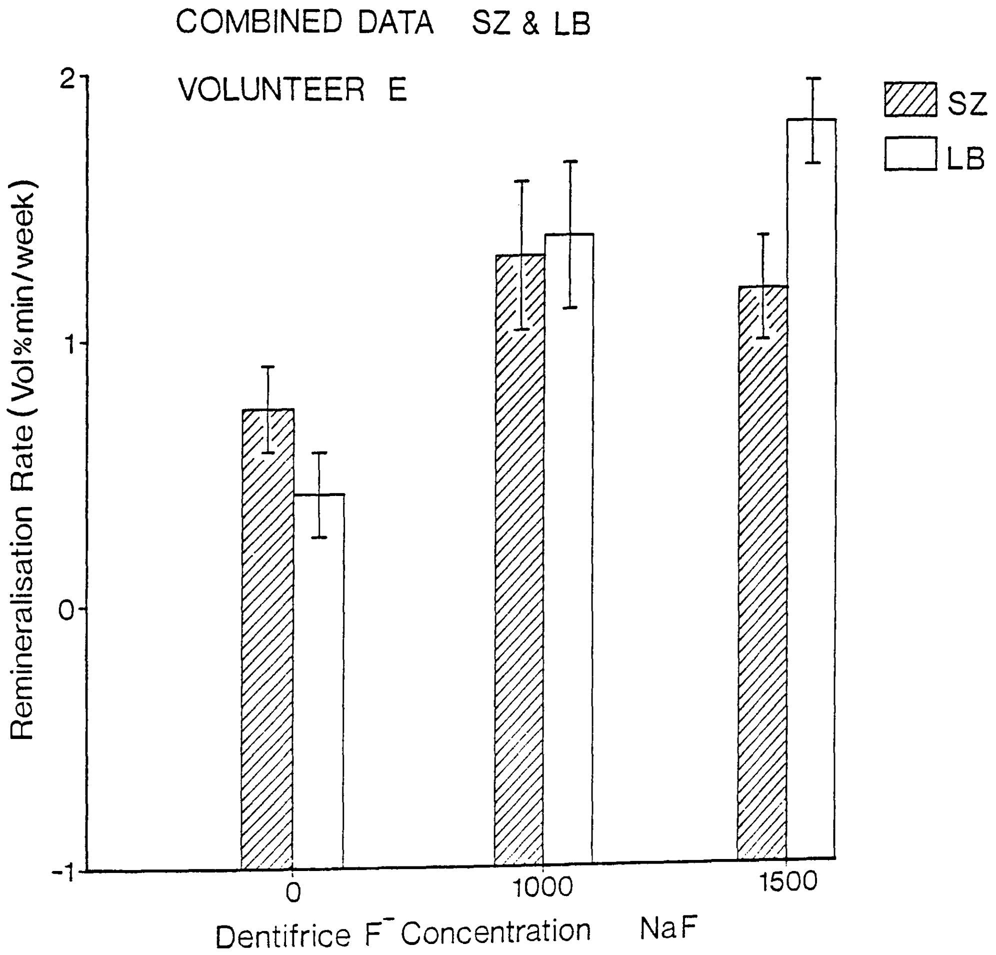

Figure 5.29 Plot of surface zone (SZ) and lesion body (LB) remineralisation rates (% volume mineral/week) against NaF dentifrice concentration for volunteer E.

TABLE 5.1 INDIVIDUALS' DMFT/DMFS, CALCULUS & SALIVARY FLUORIDE LEVELS

Volunteer	A	B	C	D	E
DMFT	18	13	15	16	11
DMFS	30	19	30	49	22
CALCULUS	0	2.5	4	7	0
B.S. Fluoride level (ppm)	0.031	0.067*	0.031	0.035	0.063
Exposed F level (ppm)	0.403	0.051*	0.185	1.200	4.550

* - control subject
B.S. - Baseline Salivary

TABLE 5.2 CROSS-OVER DESIGN NaMFP/NaF

Dentifrice Fluoride concentration (ppm)

	Volunteer	Run 1	Run 2	Run 3	Run 4
NaMFP					
	A	1000	1500	2500	0
	B	1500	2500	1000	0
	C	2500	1000	1500	0
NaF					
	D	1000	1500	0	
	E	1500	1000	0	

TABLE 5.3 TABLE OF MEAN (± 1 S.E.) OF BASELINE IML'S AND FINAL SLOPES

	F^- conc. (MFP) 0	1000	1500	2500
SUBJECT A				
No. of lesions	14	5	4	11
Interc.	48.19	26.43	33.25	34.47
± S.E.	0.78	0.56	1.85	0.46
Slope	0.18	0.48	0.88	0.36
± S.E.	0.23	0.19	0.63	0.14
SUBJECT B				
No. of lesions	15	14	5	9
Interc.	25.51	27.65	34.14	25.79
± S.E.	0.47	0.55	0.61	0.59
Slope	0.21	1.40	1.06	0.94
± S.E.	0.14	0.16	0.20	0.20
SUBJECT C				
No. of lesions	11	13	15	15
Interc.	32.96	26.28	26.90	27.0
± S.E.	0.59	0.40	0.34	0.33
Slope	0.28	0.99	1.04	1.04
± S.E.	0.18	0.14	0.10	0.11

	F^- conc. (NaF) 0	1000	1500
SUBJECT D			
No. of lesions	12	7	7
Interc.	26.31	34.37	27.56
± S.E.	0.76	0.70	0.43
Slope	-0.51	1.22	0.73
± S.E.	0.41	0.21	0.15
SUBJECT E			
No. of lesions	10	6	11
Interc.	28.07	22.0	25.10
± S.E.	0.52	0.81	0.60
Slope	0.55	1.40	1.05
± S.E.	0.17	0.27	0.18

Table 5.4 Significance between concentrations of NaMFP, for Integrated Mineral Loss (IML), Surface Zone (SZ) and Lesion Body (LB).

Between Dentifrice	IML Volunteer			SZ Volunteer			LB Volunteer		
	A	B	C	A	B	C	A	B	C
0 - 1000	NS	**	**	*	**	*	NS	**	**
0 - 1500	NS	*	**	NS	**	**	*	*	**
0 - 2500	NS	*	**	NS	*	**	NS	NS	**
1000 - 1500	NS	NS	NS	*	NS	NS	*	NS	NS
1000 - 2500	NS	NS	NS	NS	NS	**	NS	NS	NS
1500 - 2500	NS	NS	NS	NS	NS	**	**	NS	NS

Legend: NS = Not Significant (P < 0.05)

* = 0.01 < P < 0.05)

** = P < 0.01

Table 5.5 Significance between concentrations of NaF for Integrated Mineral Loss (IML), Surface Zone (SZ) and Lesion Body (LB).

Between Dentifrice	IML		SZ		LB	
	D	E	D	E	D	E
0 - 1000	*	*	NS	*	*	**
0 - 1500	*	*	*	*	*	*
1000 - 1500	NS	NS	*	NS	NS	Δ

Legend:

NS = Not Significant ($P > 0.05$)

* = $0.01 < P < 0.05$

** = $P < 0.01$

Δ = $P < 0.05$

CHAPTER 6 THE DEVELOPMENT OF A NEW *IN VITRO* MODEL

6.1 INTRODUCTION

As discussed in detail in Chapter 1, enamel remineralisation studies have been carried out either *in vitro*, or *in vivo*/*in situ*. The *in situ* technique proposed in this project has the major advantage that changes in lesion mineral content, which occur within the natural oral environment, can be measured. However, like all *in situ* techniques, it suffers from the disadvantage that the experiments are time-consuming and only a single parameter can be studied in one experiment. The development of a more accurate *in vitro* model which would make an accurate prediction of *in vivo* results, would overcome this drawback in that many parameters affecting remineralisation could be studied in a relatively short time-scale. A pH cycling regime has been proposed (ten Cate & Duijsters, 1982), in which sections were alternated between demineralisation and remineralisation solutions in an attempt to mimic the changing pH of the enamel/plaque interface zone. It was concluded, however, that a situation was achieved whereby an enamel lesion could neither be de- nor remineralised further. The authors did not propose an explanation for this result, but merely reported their observations. It is possible that calcium fluoride, which is known to form under similar *in vitro* remineralising conditions had, in the main part, been removed, leaving a fluoridated mineral which resisted

dissolution by the acid.

The choice of mode, duration and frequency of the further acid attack, however, has been empirical (Chapter 1). The aim of this part of the study was to develop an *in vitro* cycling model to study the NaMFP dentifrices used previously in the *in situ* studies (Chapter 5). It was decided, for practical reasons, that the daily *in vitro* regime be split into two parts. In one part the sections would be exposed to an overnight (16 hrs) acid attack and in the other subjected to the test slurry for a brief period and then stored in an artificial saliva.

In the first part of this chapter, the choice of a suitable acid attack will be described, the overnight pH of which was determined by comparing demineralising rates of sound enamel sections *in vitro* with those found *in situ*. In the second part of the chapter, the *in vitro* study with the three NaMFPP dentifrices will be presented.

6.2 FURTHER ACID ATTACK

6.2.1 Demineralisation rate - *in situ*

The enamel section carrying appliances previously described in Chapter 4 were again employed in this part of the project. Five volunteers participated and each was given an appliance housing four enamel sections from sound human premolars, extracted for orthodontic reasons, as described in Chapter 2. Microradiographs of the sections

were taken prior to commencement of the experiment, firstly to act as baseline controls and secondly, to ensure that no incipient enamel lesions were aleady present on the teeth. The participants were given a non-fluoridated dentifrice a minimum of 1 week before the start of the procedure, and were asked to refrain from using any other dentifrice or fluoride preparation. Details of the protocol "Instructions to Appliance Wearers" (see Appendix IV), on the oral hygiene procedures to be practised during the experiment were distributed. At the end of each week the sections were removed from the appliances and radiographed. The experiment lasted for 3 weeks.

6.2.2 Demineralisation rate - *in vitro*

Sections were prepared as described previously (Chapter 2) and were subjected to a daily routine of 8 hr storage in an artificial saliva (Birkeland, 1973) at 37°C, followed by 18 hr in an 10 % acidified gel at 20°C. The composition of both the artificial saliva and the acidified gel are detailed in Appendices I & X. Experiments were carried out over a range of gel pH values between pH 4.0 and pH 5.0. The gel pH was adjusted by the addition of 1 M lactic acid (Chapter 2). Three sections were allocated to each pH group and the experiments continued for 14 - 30 days depending on the gel pH. At intervals during the experiment, again depending on the gel pH, the varnish was removed from the sections which were then radiographed. Microdensitometric tracings of

the lesions and aluminium stepwedges for both _in situ_ and _in vitro_ parts of this project were obtained using the Joyce-Loebl 3CS microdensitometer, as described in Chapter 3. A slit width of 10 μm and length 100 μm was, however, employed when scanning both the lesion and the wedge. Mineral content values were calculated as described previously (Section 2.5).

6.2.3 Results

Typical radiographs of lesions created _in situ_ and _in vitro_ are shown in Fig 6.1 and 6.2 respectively. In the _in situ_ studies only three out of the twenty sections, each from different volunteers, showed areas of demineralisation, whereas all _in vitro_ sections subjected to pH < 4.8 demineralised. In both the _in situ_ and _in vitro_ studies the lesions formed were not homogeneous. Thus, for each demineralised section, microdensitometric tracings were made at the site of maximum mineral loss. The progression of a lesion created _in vitro_ at pH 4.4 is shown in Fig 6.3. The change in IML between the baseline and subsequent tracings is shown in Fig 6.4 for the three _in situ_ sections which demineralised and in Fig 6.5 for the _in vitro_ sections at the different pH values. The mean ($\pm$ 1 S.D.) demineralisation rate for the three _in situ_ sections was 1.19 ($\pm$ 0.2) μm/wk. For the _in vitro_ sections, a least squares fit was performed on the plots of demineralisation rate against hydrogen ion concentration (Fig 6.6) and the pH value calculated which corresponded to the mean demineralisation rate found in

the *in situ* studies. Thus, from these results, an overnight exposure to an acidified gel at pH 4.7 gave a demineralisation rate similar to the maximum obtained with these five volunteers.

6.2.4 Discussion

The aim of this part of the study was to determine a practical daily acid attack regime for use in *in vitro* remineralising experiments which would give demineralising rates similar to those found in the oral environment. The results suggest that a daily 16hr acid attack in gel at pH 4.7 gives a demineralisation rate of the order found *in situ*. A similar result has been found previously by ten Cate & Duijsters (1982).

There are several limitations to this study as *in vivo* de- and remineralisation rates cannot be represented by a single value and there will be, as previously stated in Chapter 5, large person-to-person variations (Featherstone *et al.*, 1982; Mellberg *et al.*, 1985; Corpron *et al.*, 1986; Dijkman *et al.*, 1986). As a result, remineralisation experiments, both *in vitro* and *in situ*, should be carried out over a range of acid attacks. Furthermore, the volunteers used in the *in situ* study were all dentally aware and, as such, the demineralisation rates obtained may not reflect those of a wider population. However, by using only the most demineralised areas for comparison this factor can be partially offset. While only one section was studied at each of the *in vitro* pH values,

the good correlation between demineralisation rate and hydrogen ion concentration would suggest that the errors involved in determining the equivalent pH value would be minimal, especially when the limitation of using a single acid attack regime is borne in mind. In agreement with ten Cate & Duijsters (1982), the results of this study provide a basis for the choice of daily acid attack for incorporation into *in vitro* remineralisation studies.

6.3 A PILOT STUDY INCORPORATING FURTHER ACID ATTACK WITH FLUORIDE EXPOSURE

6.3.1 Methods and materials

Artificial enamel lesions were created *in vitro* in sound human premolars, as described in Chapter 2. Each tooth was immersed in 10ml of gelatin acidified with 1M lactic acid to pH 4.0 for a period of 10 weeks. Six or seven longitudinal sections were cut and ground from each tooth. Baseline mineral content measurements were made and the sections varnished on all cut aspects apart from their natural enamel surfaces. Four sections were allocated to one of six groups. The sections in Group 1 were stored in an artificial saliva (Birkeland, 1973) at 37°C. Sections in Groups 2 - 6 were kept in artificial saliva for 8 hrs and exposed to a daily acid attack (acidified gel, pH 4.7, 37°C) for 16 hrs. In addition, sections in Groups 3, 4, 5 and 6 were exposed twice daily for 2 min to 20 % slurries of non-F, 1000, 1500, 2500 ppmF (NaMFP) dentifrices respectively. The experiment was continued for five weeks

with microradiographic/ microdensitometric analysis of mineral content carried out every five days (for details see Chapter 2). For this part of the study, the Leitz ASBA microdensitometer was available and, therefore, for convenience and speed, the analysis was carried out on this unit.

6.3.3 Results

The only group showing a net demineralisation was the set of lesions in Group 2. Here, the slope of the least squares fit increased showing that, on average, the lesions exposed to 8 hrs artificial saliva plus 16 hrs acid attack, demineralised at a rate of 0.21 ($\pm$ 0.21) μm/wk. The mean de- / remineralisation rates for the different groups are shown in Fig. 6.7. The group exposed solely to the artificial saliva (Group 1), showed the greatest remineralisation, whereas lesions exposed to the overnight acid attack (Group 2) demineralised, as stated previously. The addition of twice daily section exposure to dentifrice slurries (Groups 3 - 6), although resulting in minimal remineralisation, did reverse the demineralising trend of the acid attack. Statistical analysis was carried out using a t-test and showed that no obvious differences in remineralisation rates were found for any of the Groups exposed to the different dentifrice slurries.

6.4 DISCUSSION

Although _in situ_ techniques have the advantage of being able to study enamel remineralisation in the natural oral environment, only a few samples and a single parameter, eg. fluoride concentration, can be accommodated in a single experiment. The development of an _in vitro_ model which would predict accurately _in vivo_ remineralisation would enable large numbers of experiments to be carried out. It has been suggested by ten Cate & Duijsters (1982), that incorporation of a further acid attack would improve the reliability of _in vitro_ studies, when attempting to mimic the clinically reported inhibition of remineralisation in low fluoride areas. In this study, circumstances were fortunate in that direct comparison could be made between the results of an _in vitro_ model incorporating a daily acid attack with those of the _in situ_ study of Chapter 5, using the same dentifrices. The enamel remineralisation rates with the non-F dentifrice were similar in both studies. However, in contrast with the _in situ_ study, the _in vitro_ remineralisation rates for the fluoride-containing dentifrices were not signicantly different from the non-F dentifrice. One possible explanation for this observation could be that the acid attack used in the _in vitro_ study was too severe. In the first part of this Chapter, comparison was made between the demineralisation rates of sound enamel whereas, in the pilot remineralisation study the enamel had been previously demineralised. It is possible that the two situations are not comparable, since it has been shown

recently that artificial lesions and sound enamel, when subjected to the same acid attack have different demineralisation rates (Damato et al., 1987). In addition, the chemistry of the apparent availability of free fuoride from the sodium monofluorophosphate is a complex situation and is believed to be only fully "activated" in the oral environment, in the presence of salivary phosphatases (Mellberg & Mallon, 1984). However, the results are in general agreement with those recently reported by other workers using NaMFP dentifrices (ten Cate & Rempt, 1986), and the technique deserves further investigation.

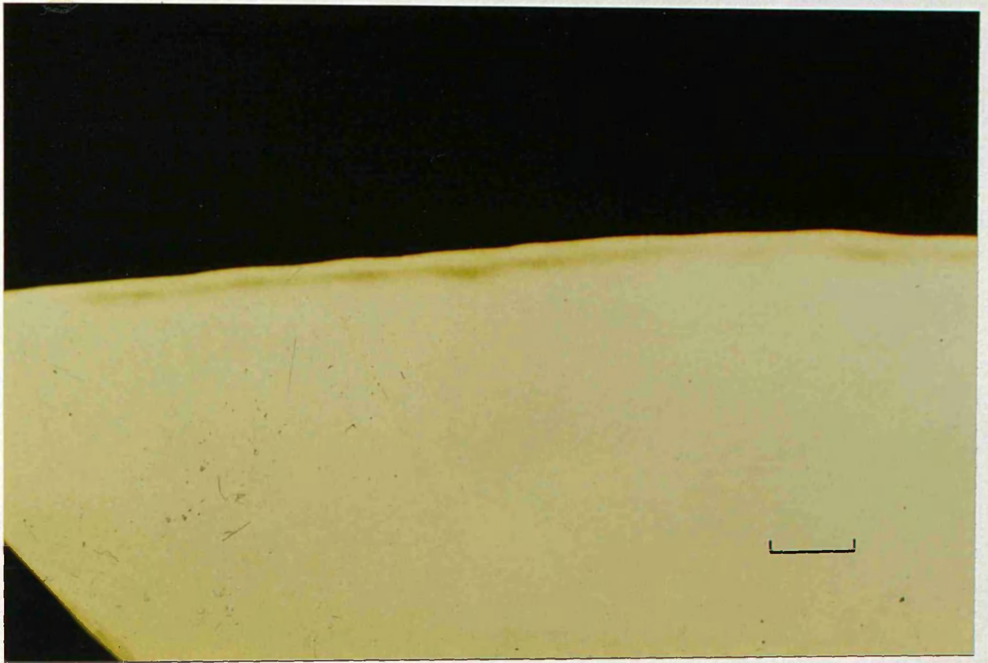

Figure 6.1 Microradiograph of an early enamel lesion created *in situ* after 3 weeks' intraoral exposure to a non-fluoridated dentifrice. Bar is 100 μm.

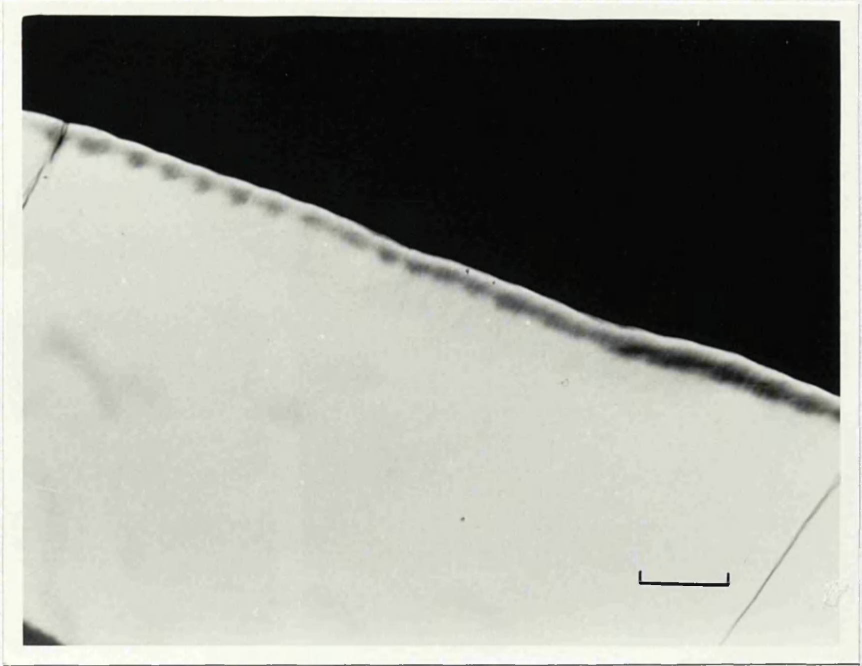

Figure 6.2 Microradiograph of a subsurface lesion created *in vitro* after 3 weeks' exposure to an acidified gelatin (pH 4.6). Bar is 100 μM.

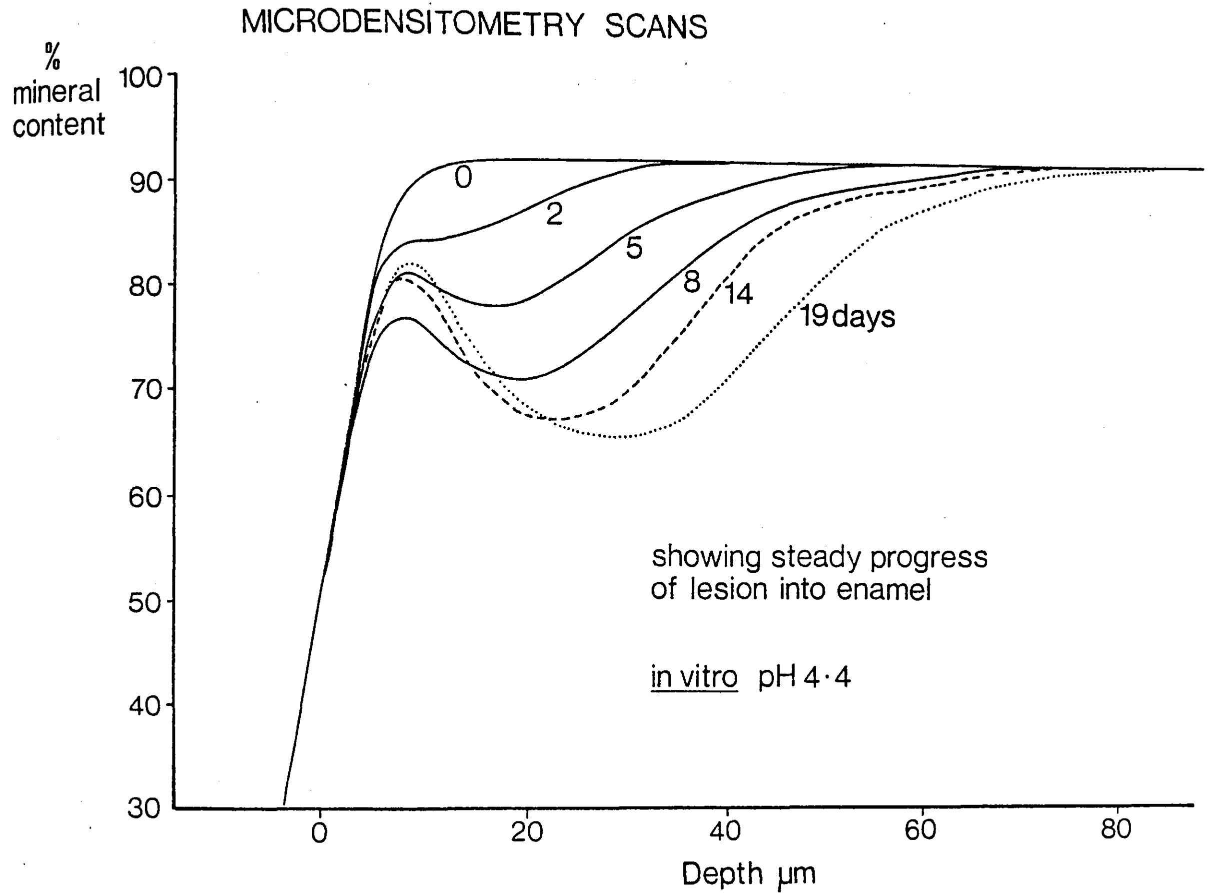

Figure 6.3 Successive microdensitometric scans of an in vitro lesion created at pH 4.4.

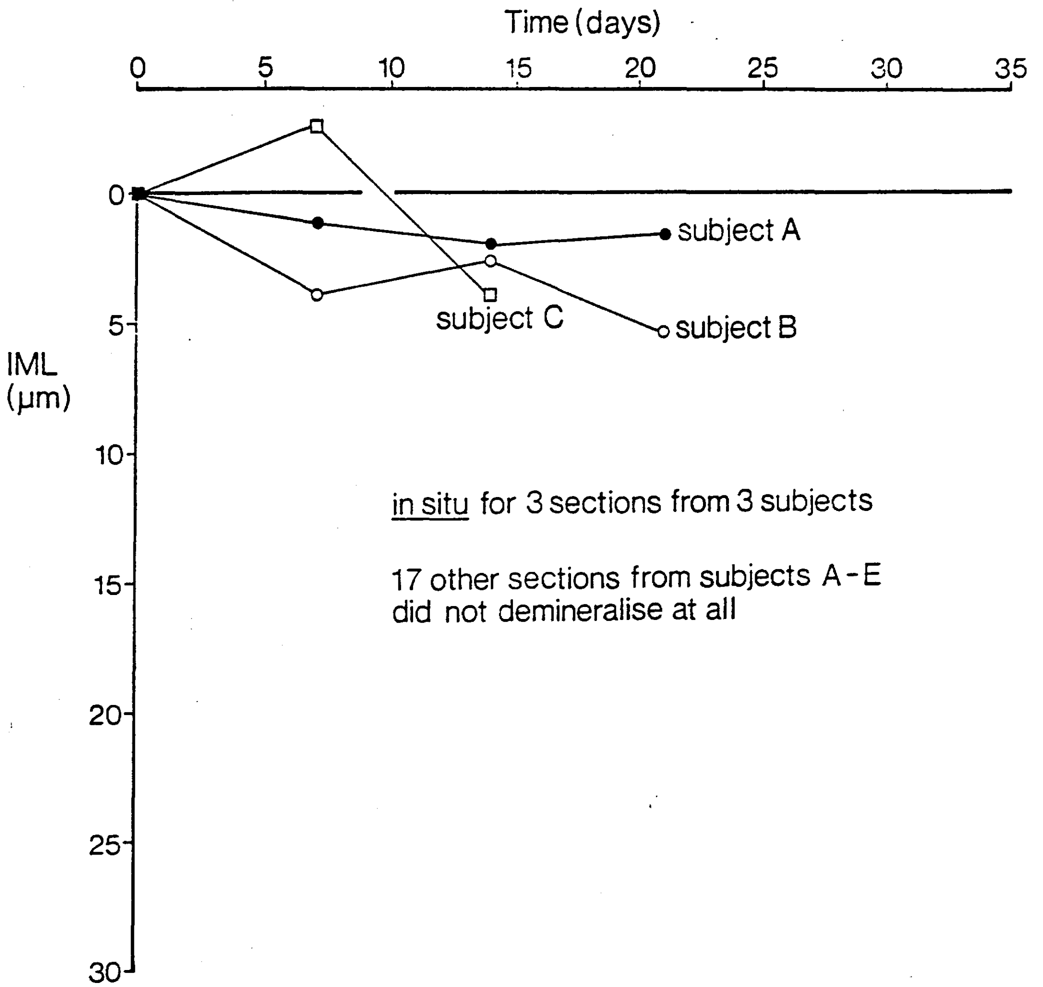

Figure 6.4 Plot of the change in integrated mineral loss (IML), from baseline, for the three in situ created lesions

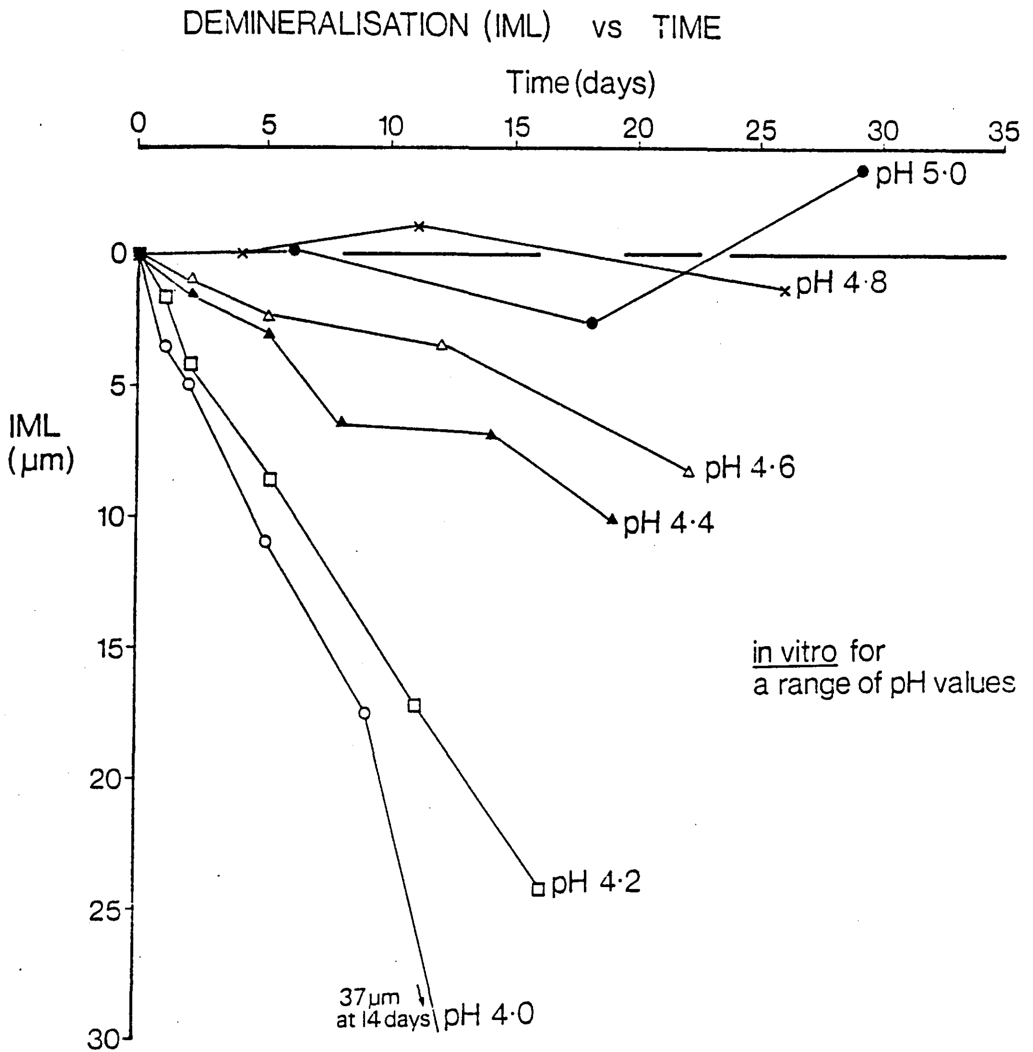

Figure 6.5 Plot of the change in integrated mineral loss (IML), from baseline, for the range of <u>in vitro</u> created lesions.

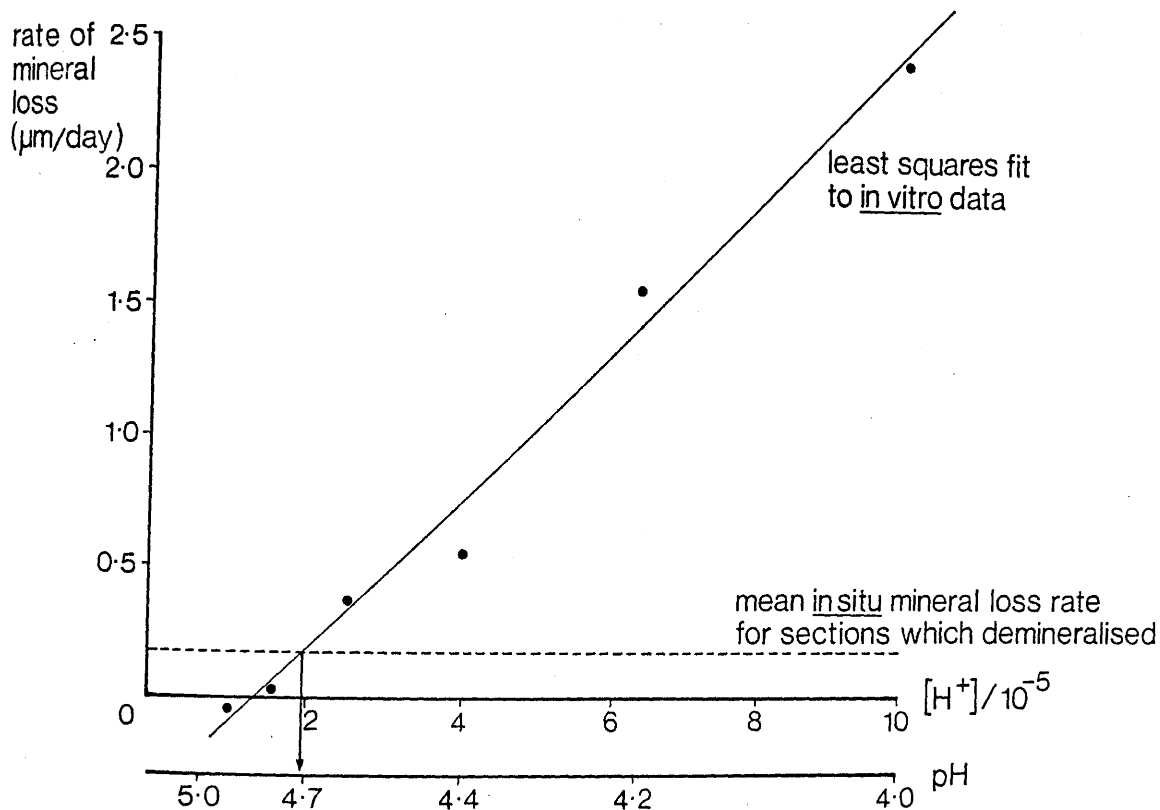

Figure 6.6 Plot of the least squares fit to the *in vitro* demineralisation rate, versus the hydrogen ion concentration.

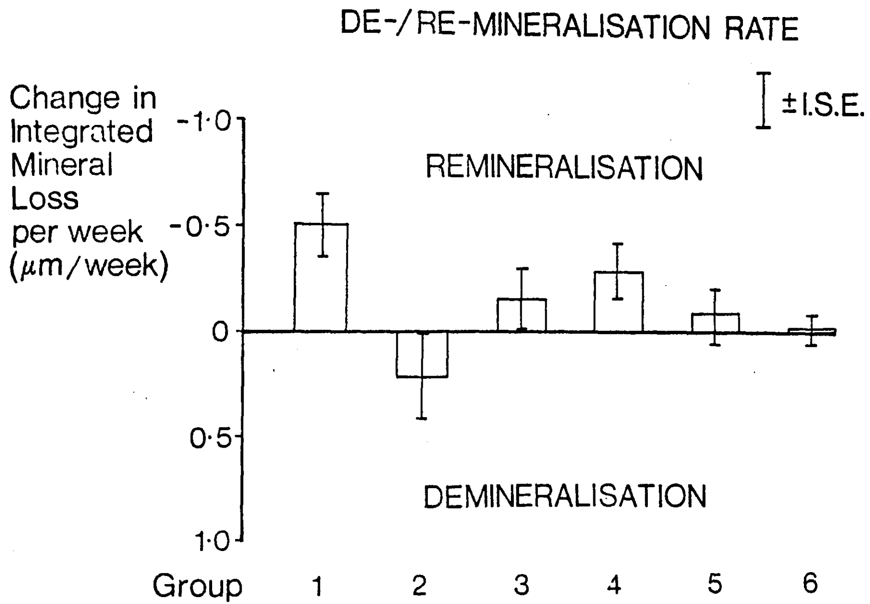

Figure 6.7 Plot of the mean de-/remineralisation rates for the different groups 1 - 6 (± 1 S.E.).

CHAPTER 7 CONCLUSIONS AND DISCUSSION

The aims of this project as detailed in Chapter 1 were as follows:

1. to design and develop a new in situ caries-investigative method,

2. to carry out an in situ remineralisation study using dentifrices of differing fluoride concentrations,

   - and -

3. to develop an in vitro model to simulate some of the conditions of the enamel/plaque interface zone.

The first aim has been fulfilled. The in situ appliance developed in this project coupled successfully the benefits of an in vivo approach along with the advantages of the single section technique discussed in Chapter 1. The major advantage of the system is the ability to assess the mineral content of the same lesion throughout the study resulting in an increase in sensitivity to small changes in mineral content. In addition, the studies in Chapter 4 showed that the plaque which formed on the enamel sections was not significantly different from the plaque on an adjacent natural enamel surface. This indicated that the test trough produced an environment similar to that of a natural interproximal site. Throughout all the studies in this thesis the appliances were well tolerated by the volunteers and no major

problems were encountered in the frequent handling of sections. However, the single section technique has the major disadvantage of requiring a great deal of care when varnishing specimens, to ensure the nail polish is right up to the edge of the cut surface of the section. At all times, the control of varnish flow is critical (a) during experimental window preparation, (b) when accurate section coverage is necessary, and (c) during placement of the specimens on to the trough area. However, with care the advantages of the technique can be attained.

So far as the second aim was concerned, the *in situ* appliance successfully facilitated the remineralisation experiment reported in Chapter 5. However, when comparing remineralisation rates of the different dentifrices, the results of this study were in contrast to the observed *in vivo* concentration effect reported by Stephen *et al.* (1987). In that study the same three NaMFP dentifrices were used in a clinical caries trial involving over 3000 school children. Whilst it was hoped that the results of this clinical trial would be predicted by the *in situ* project, the possible explanations for this discrepancy are discussed below.

1. The number of volunteers in this project was low and certainly, in future tests, a greater number of individuals would be involved. However, the subjects involved in this project were all dentally aware and all adhered strictly to the protocol of the experiment. However, as a result of their high standard of oral

hygiene and low cariogenic diet, it would appear that the maximum remineralisation potential of these dentifrices may have already been achieved by 1000 ppmF. Thus, the influence of the diet is considered to be of prime importance, the carbohydrate intake of a typical 12 - 14 year old schoolchild inevitably being higher and more frequent than that of a well-motivated professional dental school member of staff. This is further substantiated by the clinical trial finding that the concentration effect was more pronounced in those subjects with a high initial caries prevalence (Stephen et al., 1987), in contrast with the in situ volunteers, the general lack of further demineralisation of those lesions exposed to the non-fluoridated dentifrice would support a low level of caries activity in these subjects.

2. In light of recent research, the acidified gelatin lesion creation method, may not have been the method of choice for the remineralisation experiment. The influence of trace fluoride, for example, has now been well documented (Borsboom et al., 1985) and as such, significant remineralisation may have already occurred during the 10 - 12 week lesion creation period. If this were the case, then the partly "remineralised" lesions would only demineralise further in the most severe circumstances, a situation not likely to occur in this well-motivated panel of subjects.

3. It is possible that a different stage in the carious process is being observed in this study compared to that of Stephen *et al*. (1987). The lesions in the *in situ* study were small and perhaps more susceptible to remineralisation, while the epidemiological observations of Stephen *et al*. (1987) may have resulted from remineralisation of lesions in a more advanced stage. It would also be worthwhile extending the *in situ* remineralisation experiments over a longer period than 5 weeks, to investigate if there are differences in the time courses of remineralisation with different concentrations of fluoride. The most obvious reason must, however, still be the dental awareness of the subjects used in this project.

Despite the discrepancy mentioned above, the significantly enhanced remineralisation in those lesions subjected to a fluoridated dentifrice compared to a placebo, is in agreement with other workers (Featherstone *et al*., 1982; Mellberg *et al*., 1985; Corporon *et al*., 1986). Like those cited above, some person-to-person variation was noted, although here this was only evident during the use of the fluoridated dentifrices and not the placebo. Indeed, there was remarkable reproducibility in the remineralisation rates of all volunteers when using the non-fluoridated dentifrice. It is not clear, however, whether further demineralisation would have occurred had the lesions been either exposed to a further acid attack challenge (in the form of external sucrose exposure) or had the lesions been created using a different method. The addition of both of the above alterations to the

protocol may have resulted in a concentration effect, and is worthy of further investigation.

Finally, with regard to the third aim, the results of the pilot <u>in vitro</u> study were of considerable interest. In agreement with the <u>in situ</u> study, the <u>in vitro</u> test failed to differentiate between the three concentrations of NaMFP, but in addition also failed to demonstrate an increased remineralisation rate of the fluoridated dentifrices when compared with the non-fluoridated placebo. There is little doubt that additional work in this model is necessary, certainly with regard to both the further acid attack and the initial lesion creation method which, in light of recent studies can both be improved. To ensure a complete absence of fluoride, both in the initial lesion creation method and in the further acid challenge, a buffered solution method ought to be employed. In addition, the duration of lesion exposure to the further acid challenge was a little more severe than was anticipated. Thus, pilot experiments using solutions were initiated and the early results are encouraging.

As a final appraisal of the technique, it would appear to have a place in the further investigation of fluoride's role in lesion remineralisation. In common with all remineralisation techniques, the choice of lesion creation method and the use of either abraded or nonabraded enamel is open to debate and merits further investigation. However the work presented in this thesis has shown that the <u>in situ</u> technique is ideal for investigating not

only the parameters pertaining to a fluoride regime, but also those relating to other modalities. The *in situ* remineralisation study has shown in detail mineral redeposition over a 5 week period. One result of that study is that it would be possible to determine, without loss of accuracy, the remineralisation rate with fewer microradiographs. This would greatly reduce the effort involved, and allow greater number of volunteers to be employed in future studies. While, work is also currently underway investigating the remineralising effects of mouthrinses, the *in situ* device is being utilised in studies designed to investigate the remineralisation of root caries as well as for the microbiological characterisation of organisms commonly associated with the carious lesion.

APPENDICES

APPENDIX I - THE ACIDIFIED GELATIN GEL

Source: Difco Laboratories

Composition:		Amount
	Gelatin gel	100 g
	Tricalcium phosphate	0.17 g
	Lactic acid (1M)	to pH
	Distilled water	900 ml

Method of Preparation:

1. The gelatin was dissolved in 800 ml of distilled water at 100°C.
2. The tricalcium phosphate was dissolved in 100 ml of distilled water and added to the gelatin solution, thoroughly mixed and allowed to stand for 24 hr.
3. The gelatin was reheated to 37°C and the lactic acid added to the required pH and allowed to stabilise for 1 hr.
4. The acidified gelatin was then poured into 20 ml vials (Taab Laboratory Equipment Ltd., Reading, England).
5. The gelatin was reheated to 37°C for immersion of the prepared tooth and stored at room temperature.

APPENDIX II - DERIVATION OF THE EQUATION BY ANGMAR *ET AL.* (1963)

The grey level for any point in the lesion, which has resulted from X-ray absorption by both organic and inorganic components (thicknesses t_m and t_o respectively), can be equated against an equivalent aluminium (Al) grey level. Hence, for a particular level of absorption of X-rays (ie. the grey value of a part of the lesion), the absorption can be equated against the absorption in an aluminium stepwedge.

Thus, $\mu_a t_a = \mu_m t_m + \mu_o t_o$

where,
- μ_a = linear absorption coefficient of the aluminium
- μ_m = linear absorption coefficient of the mineral component
- μ_o = linear absorption coefficient of the organic component
- t_m = thickness of the mineral element
- t_o = thickness of the organic element
- t_a = equivalent thickness of aluminium to give that grey value

but, $t_s = t_m + t_o$, where t_s = section thickness

and $V_m/V_s = t_m/t_s$

where,
- V_m = volume of mineral component
- V_s = section volume

thus,

$$V_m/V_s \times 100 = 100 \frac{(\mu_a t_a - \mu_o t_o)}{(\mu_m - \mu_o) t_s}$$

The absorption coefficients depend on the radiation source (eg. kV, target, filter, etc.). Angmar *et al*. (1963) employed CuK α radiation; μ_a, μ_m and μ_a were found from known data and thus the equation reduces to:

$$\% \text{ vol. min.} = \frac{52.77\, t_a}{t_s} - 4.54$$

Thus, the only unknown is t_a, since t_s can be measured (section 2.4). Therefore, for every point in the enamel, the equivalent aluminium thickness (t_a) is derived and percent volume mineral calculated.

APPENDIX III

GREATER GLASGOW HEALTH BOARD

225 BATH STREET
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Telephone: 041-204 2755

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....

If 'phoning ask for:— Mr R. McKechnie

9 August 1984

Dr. K.W. Stephen
Reader in Oral Medicine and Pathology
Glasgow Dental Hospital and School
378 Sauchiehall Street
GLASGOW
G2 3JZ

Dear Dr. Stephen

AREA DENTAL ETHICS COMMITTEE

I write to inform you that your application of 6 August 1984 for clinical research has now been discussed by the Chairman of the Area Dental Ethics Committee, the aim of the project being:

"Investigations Relating to Clinical Topical Fluoride Therapies"

This project has now been approved and you may proceed with your research. The decision will not be minuted until the next formal meeting of the Area Dental Ethics Committee.

The Committee would be grateful if you would inform them of the results of your project and any ethical problems encountered whenever the project is completed.

Yours sincerely

R. McKechnie

R. McKECHNIE
Chief Administrative Dental Officer

c.c. Mr H.A. Critchlow, Chairman
Mr G. Lewis, Secretary

APPENDIX IV - INSTRUCTIONS TO APPLIANCE WEARERS

1. The appliance should be worn for at least 1 week prior to its use in the research programme, to allow any irritating areas to be recognised and altered accordingly.

2. Each phase of the experiment will last for 1 week (7 days).

3. The diet should be normal with no particular emphasis on any type of food.

4. A coded supply of toothpaste will be supplied and this should be used in place of your regular brand. No other fluoride supplement should be used during the experiment. In addition, please continue to use the test dentifrice even when not taking part in the procedure.

5. Oral hygiene should be practised in the morning and in the evening only and in the following manner:

(a) Place a uniform thickness of toothpaste along the length of the Oral B 35 toothbrush.
(b) Remove the appliance and leave it undisturbed in a safe place. This will allow the lingual aspects of the lower arch to be cleansed (with floss, etc.).
(c) Reinsert the appliance and brush all other aspects of the dentition for a period of 2 min.
(d) Do not rinse out at the end of the procedure.

6. At the end of the week the appliance should be placed in the polythene bag provided with a damp gauze and returned to Steve Creanor as soon as possible.

APPENDIX V - TRYPTICASE SOY BLOOD AGAR (TSBA)

Source: Gibco Bio-Cult Diagnostics Ltd.

Composition:		Amount
	Tryptic Soy Agar (TSA) base*	40 g
	Vitamin K/Haemin	10 ml
	Defibrinated horse blood	50 ml
	Distilled water	1000 ml

Method of Preparation:

1. The TSA base was dissolved in the distilled water at 100°C.
2. The pH was adjusted to 7.3.
3. The media was then autoclaved at 121°C for 15 min and allowed to cool to 56°C.
4. The Vitamin K/Haemin and the defibrinated horse blood was added and allowed to mix thoroughly.
5. Finally, the agar was poured into sterile 90 ml Petri dishes in 15 ml volumes.

* Composition of the Tryptic Soy Agar (TSA) base:

	g/1000 ml
Peptone 140	15
Peptone 110	5
Sodium chloride	5
Agar	15

APPENDIX VI - MITIS SALIVARIUS BACITRACIN AGAR (MSB)

Source: Difco Laboratories

Composition:		Amount
	Mitis Salivarius Agar (MSA) base*	90 g
	Sucrose	150 g
	1 % Bacto Chapman Tellurite	1 ml
	Bacitracin	20 units
	Distilled water	1000 ml

Method of Preparation:

1. The Mitis Salivarius Agar base and the sucrose were added to the distilled water at 100°C.
2. The pH was checked to be 7.3 and the media was autoclaved in 500 ml bottles at 121°C for 15 min.
3. When required, the agar was melted, cooled to 45°C and 1 ml of 1 % Bacto Chapman Tellurite (Difco) added.
4. 10 ml of a sterile solution containing 20 units of Bacitracin (Sigma Chemical Company) per ml were added to 1000 ml of media.
5. The media was poured into sterile 90 ml Petri dishes in 15 ml volumes.

* Composition of Mitis Salivarius Agar (MSA) base:

	g/1000 ml
Bacto-Tryptose	10
Proteose Peptone No. 3	5
Proteose Peptone	5
Bacto-Dextrose	1
Saccharose	50
Dipotassium phosphate	4
Trypan blue	0.075
Bacto-crystal violet	0.0008
Bacto-Agar	15

APPENDIX VII - ROGOSA SL AGAR

Source: Difco Laboratories.

Composition:		Amount
	Rogosa Agar base*	75 g
	Glacial acetic acid	1.32 ml
	Distilled water	1000 ml

Method of Preparation:

1. The Rogosa Agar base was allowed to dissolve in the distilled water at 100°C.
2. The glacial acetic acid was added, the pH was checked to be 5.4 and the media was boiled for a further 2 - 3 min.
3. After cooling the media was poured into sterile 90 ml Petri dishes.

* Composition of Rogosa Agar base:

	g/1000 ml
Bacto-Tryptone	10
Bacto-yeast extract	5
Bacto-dextrose	10
Bacto-arabinose	5
Bacto-saccharose	5
Sodium acetate	15
Ammonium citrate	2
Monopotassium phosphate	6
Magnesium sulphate	0.75
Manganese sulphate	0.12
Ferrous sulphate	0.03
Sorbitan monooleate	1
Bacto-Agar	15

APPENDIX VIII - PHOSPHATE BUFFERED SALINE

Source: Flow Laboratories

Composition:		Amount
	Sodium chloride	8 g
	Potassium chloride	0.2 g
	Disodium hydrogen phosphate	1.15 g
	Potassium dihydrogen phosphate	0.2 g
	Distilled water	1000 ml

Method of preparation:

1. 10 tablets were added to the distilled water and allowed to dissolve thoroughly.
2. The pH was checked to be 7.3, the solution was dispensed into universal containers and autoclaved at 121°C for 15 min.

Appendix IX — Formulation of Test Toothpastes

	NaF/Silica 1000/1500 ppmF	Silica Placebo	NaMFP/Alumina 1000/1500/2500 ppmF	Alumina Placebo
Abrasive	Silica 22%	Silica 22%	Alumina 50%	Alumina 50%
Humectant	Sorbital 50%	Sorbital 50%	Sorbital 27%	Sorbital 27%
Thickener	SCMC 0.65%	SCMC 0.65%	SCMC 0.85%	SCMC 0.85%
Therapeutic Agent	NaF 0.22%/0.33%		NaMFP 0.76%/1.14%/1.90%	
Sweetener, Flavour etc. & Water	←——— 100 % ———→			

SCMC Sodium Carboxymethylcellulose
SLS Sodium lanrylsulphate

APPENDIX X - THE ARTIFICIAL SALIVA (BIRKELAND, 1973)

Source: B.D.H.

Composition:		concentration
	$CaCl_2$	1×10^{-3}M
	NaH_2PO_4	3×10^{-3}M
	$NaHCO_3$	2×10^{-2}M

Method of Preparation:

1. The components of the saliva were added to 1000 ml of distilled water at 100°C.
2. For 24 hours the solution was mixed using a magnetic stirrer, and stored at -4°C until required.

APPENDIX XI

Basic Sciences

Caries Res. *20:* 385–391 (1986)

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In situ Appliance for the Investigation of Enamel De- and Remineralisation

A Pilot Study

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Key Words. Caries, artificial · Caries, in vivo model · Enamel, sections · Demineralisation · Remineralisation · Fluoride · Microradiography · Appliance, intra-oral

Abstract. A novel lower removable acrylic appliance to investigate the pathogenesis of the incipient carious lesion in enamel is described. Pilot enamel demineralisation and remineralisation studies have been undertaken. Ground enamel sections were cut from undemineralised enamel or from teeth on which artificial lesions had been created. These were varnished and mounted on the base of experimental troughs in an appliance and subjected to the natural oral environment of a volunteer's mouth. The individual's diet was not altered but an F or non-F dentifrice was used as dictated by the experimental protocol. Microradiographs of the tooth sections were taken at baseline and at weekly intervals. In the demineralisation study, 3 sections out of 20 showed areas of demineralisation after only 2 weeks, whereas in the remineralisation study an increase in the mineral content of the surface zone was evident in 3 out of 4 sections after the same period, but in all sections after 5 weeks.

Remineralisation of the early carious lesion in human dental enamel has been studied in vitro [ten Cate and Duijsters, 1982; Tyler and Poole, 1984], in situ [Koulourides et al., 1974] and in vivo [Featherstone et al., 1982; Mellberg and Chomicki, 1983; Holmen et al., 1985a, b]. Koulourides et al. [1974] developed a technique in which enamel slabs, mounted in the buccal flanges of lower dentures, were exposed to the natural oral environment. However, the use of enamel slabs has two major disadvantages: (i) the mineral content of the enamel under study can only be measured at the end of the experiment and (ii), variations in mineral content of demineralised enamel exist even between sections taken from the same tooth [Strang et al., 1985].

Harvey et al. [1982] and Featherstone and Silverstone [1982] overcame these problems in vitro by using enamel sec-

tions, the mineral content of which could be measured at any point during the remineralisation period and related to the baseline value.

An intra-oral appliance for enamel de- and remineralisation studies has been developed to extend this 'section technique' to the in situ situation. This article describes the design of the intra-oral appliance to accommodate enamel sections and presents results from pilot de- and remineralisation studies. A subsequent article [Creanor et al., 1986] will present the microbiological species and the acid/anion profiles of the plaque-like material which accumulated on exposed enamel surfaces of sections mounted on the appliance.

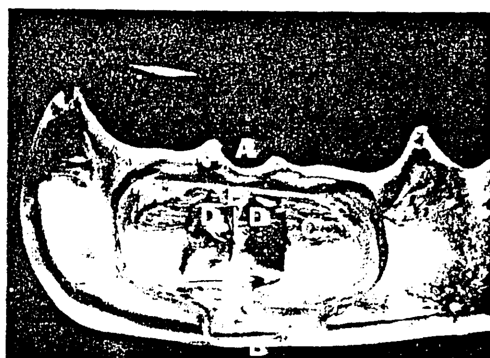

Fig. 1. One side of the appliance showing the entrance (A) and the exit (B) to the trough (C). Enamel sections (D) are mounted in a 'contact point' situation (E).

Material and Methods

The Appliance Design

A lower removable appliance (fig. 1), constructed from self-curing acrylic, was designed to fit either dentate or partially dentate lower arches. From impressions taken in alginate, casts were poured in dental stone and any undercut areas on the lingual aspect of the arch blocked out. Opposite the 76|67 interproximal areas, small pieces of wax measuring 6 × 9 × 1 mm were placed on the cast, just inferior to the contact points of these teeth. The wax was lightly warmed in tepid water, then flattened using a glass slide. The cast was duplicated and the appliance constructed on this duplicate with, on its fitting surface, troughs which had dimensions identical to the wax template. An entrance and an exit were sited on the superior and inferior aspects of the trough to allow saliva and salivary constituents to enter and leave freely through this experimental chamber. Retention of the appliance is by four half round wrought stainless steel wire clasps. Five volunteers participated in the demineralisation experiment and one in the remineralisation experiment.

Preparation of Enamel Sections

Premolar teeth, extracted for orthodontic purposes, were obtained and stored in 5% aqueous thymol solution. Prior to use, the enamel surface was cleaned with a rubber cup and a fine pumice-alcohol mixture to remove any pellicle or calculus deposits. Only those teeth with no clinically obvious incipient lesions on their buccal aspects were employed in this study. Longitudinal sections of buccal enamel were cut, using a Leitz 1600 rotating annular diamond blade, to an approximate thickness of 230 μm and were hand-ground to a final measured thickness of approximately 100–120 μm. For each appliance four sections (two for each trough) were coated using a proprietary nail varnish on all cut aspects leaving only the natural enamel surface exposed. They were then mounted on the base of the appliance troughs using the nail varnish, their non-coated surfaces being opposed to mimic a contact point as shown in figure 1. This procedure was repeated following each radiographic session (vide infra).

Microradiography and Microdensitometry

The sections were microradiographed prior to mounting on the appliance and subsequently at 7-day intervals, the varnish being removed with amyl acetate prior to exposure. Sections were mounted on Kodak high-resolution type 1A plates and were exposed at 20 kV and 40 mA for 15 min at a target specimen distance of 300 mm using an Enraf Nonius X-ray generator. The plates were developed using standard techniques.

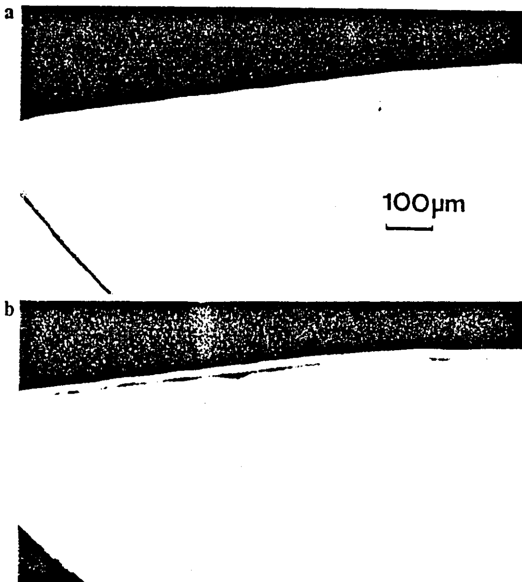

Fig. 2. Demineralisation study. Microradiographs at baseline (a) and after 2 weeks (b), of one of the sections which showed areas of demineralisation.

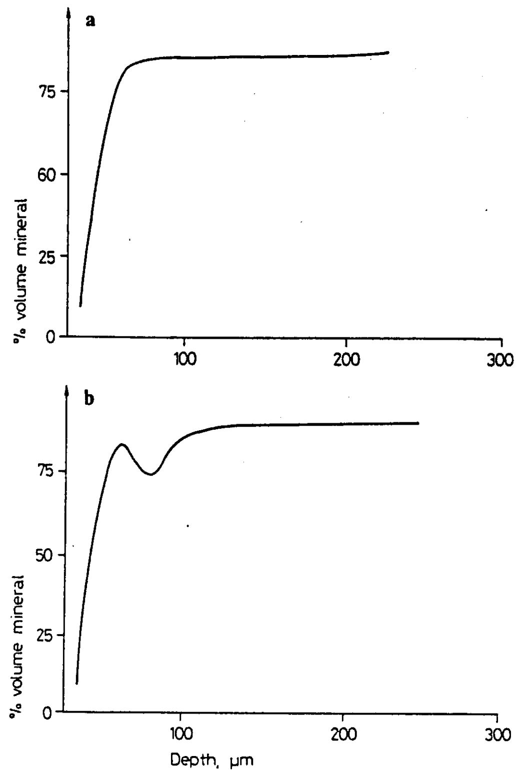

Fig. 3. Demineralisation study. Microdensitometric tracings at baseline (a) and after 2 weeks (b), from the sections in figure 2.

Microdensitometry was carried out using a Joyce-Loebl 3CS microdensitometer with a slit width of 100×3 µm. Mineral content was calculated using the formula of Angmar et al. [1963]. Measurements were made of the mineral content of the surface zone and the lesion body.

Demineralisation Experiment

Five appliances were worn for 1-week periods, during which each individual's normal dietary pattern was maintained. A non-fluoridated dentifrice was used twice daily for 2 min throughout the 2-week study period. The appliance was removed once per day to facilitate cleansing of the lower lingual aspects by tooth-brushing and flossing; thus the plaque build-up on the volunteer's natural dentition was kept to a minimum, although that which accumulated in the experimental trough was left undisturbed.

Remineralisation Experiment

For this study, sections were prepared from caries-free teeth on which artificial lesions had been created in vitro using a 10% Difco gelatin impregnated with 10 *M* lactic acid to a pH of 4.1.The lesions were of 12 weeks' duration, but otherwise the protocol was similar to that of the above demineralisation experiment apart from the use of a 1,500-ppm F (1.14% MFP) dentifrice by the volunteer twice daily for 2 min. The duration of the experiment was 5 weeks.

Results

Demineralisation

Three of the 20 sections showed areas of demineralisation with intact surface zones after 2 weeks' exposure to the non-

Table I. Microdensitometric data from the 3 sections which showed demineralisation after 2 weeks' exposure to a nonfluoridated environment

	% volume mineral			
	surface zone		lesion body	
	0 weeks	2 weeks	0 weeks	2 weeks
Section 1	79.3	66.5	78.8	63.0
Section 2	71.9	74.8	79.1	75.6
Section 3	79.3	72.4	78.0	70.9
Mean±SD	79.2±0.1	71.2±3.5	78.6±0.5	69.6±5.2

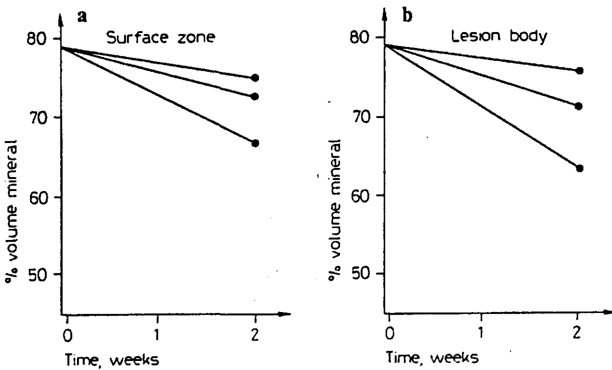

Fig. 4. Percentage volume mineral of surface zone (**a**) and lesion body (**b**) at baseline and after 2 weeks' exposure to a non-fluoridated environment.

fluoridated environment (fig. 2). The lesion sizes varied between 25 and 70 μm. The other 17 sections showed no evidence of demineralisation, although the 3 demineralised sections were from appliances worn by 3 different volunteers. Microdensitometric tracings for 1 section at baseline and after 2 weeks' are shown in figure 3, the mineral content of the surface zone and lesion body having decreased from 79.3 to 66.5% and 78.8 to 63.0%, respectively. Values for the percentage volume mineral content of the surface zone and the lesion body at baseline, and at 2 weeks, for all 3 sections are detailed in table I and shown in figure 4.

Remineralisation

Four lesions were available for analysis in this phase of the study. In 3 lesions the mineral content of the surface zone and the lesion body had increased, indicating remineralisation after 2 weeks' exposure to the fluoridated dentifrice (fig. 5, table II). After 5 weeks all 4 sections analysed showed further increases in the min-

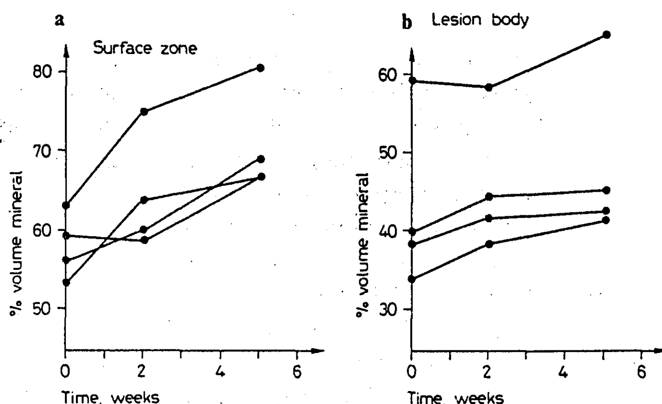

Fig. 5. Percentage volume mineral of surface zone (**a**) and lesion body (**b**) at baseline, after 2 and 5 weeks' intra-oral exposure to a 1.14% SMFP regime.

Table II. Microdensitometric data from 4 sections after 2 and 5 weeks' exposure to a 1.14% SMFP regime

	% volume mineral					
	surface zone			lesion body		
	0 weeks	2 weeks	5 weeks	0 weeks	2 weeks	5 weeks
Section 1	56.0	59.9	69.2	33.9	38.6	41.5
Section 2	59.4	59.0	66.9	59.4	58.7	65.3
Section 3	53.6	63.9	66.8	40.0	44.5	45.2
Section 4	63.1	75.2	80.4	38.6	42.0	42.5
Mean ± SD	58.0 ± 3.6	64.5 ± 6.4	70.8 ± 5.6	42.9 ± 9.7	46.0 ± 7.6	48.6 ± 9.7

eral content of the surface zone and to a lesser extent in the lesion body, the mean values of the surface zone and lesion body having increased from 58.0 to 70.8% and 42.9 to 48.6%, respectively.

Discussion

The in situ appliance described in this article overcomes the major limitations of previous in vitro and in vivo techniques. While experiments using in vitro methods are important, they do not reproduce the microbiological and biochemical conditions of the oral environment. The use of contacting enamel sections permits the caries-prone approximal areas of the dentition to be mimicked. Thus microbiological species and the acid/anion profiles of the plaque-like material accumulating at this 'contact point' can be monitored during de- and remineralisation experiments [Creanor et al., 1986].

Another major advantage of this device is that the enamel sections can be removed

at any time during the experiment to enable quantitative serial measurements of enamel mineral content to be made, obviating the need for separate control tissue with the associated reduction in sensitivity to changes in mineral content [Strang et al., 1985]. Furthermore, no ethical problems are associated with the appliance as the volunteer may remove it to allow normal oral hygiene procedures to be carried out. Thus the early decalcification induced in the natural dentition of subjects used by von der Fehr et al. [1970] and Edgar et al. [1978] does not arise. While Holmen et al. [1985a, b] overcame such ethical difficulties by utilising plaque-collecting orthodontic bands on premolar teeth destined for extraction, unfortunately their technique lacks the sensitivity of the single-section method [Strang et al., 1985].

The pilot de- and remineralisation studies have shown that measurable demineralisation of sound enamel occurs in some sections after only 2 weeks' exposure to a fluoride-free oral environment, and that significant remineralisation of artificially created lesions can occur within 2 weeks' exposure to a 1,500-ppm F (1.14% MFP) dentifrice. These early results indicate that this in situ enamel section-carrying appliance could have a significant role to play in studies of the early carious lesion and its potential for repair under a variety of controlled clinical situations. As such, more appliances have been constructed and cross-over studies are under way to eliminate person-to-person variation.

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APPENDIX XII

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Microbiology and Acid/Anion Profiles of Enamel Surface Plaque from an in situ Caries Appliance

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Key Words. Acid anions · Dental plaque · Enamel, sections · Microflora · Appliance, intra-oral

Abstract. The microbiological species and acid/anion profiles of the plaque-like material which accumulates on exposed surfaces of enamel sections mounted in the experimental troughs of the previously described in situ caries appliance were studied. Each experiment lasted 1 week and the volunteers' diet and oral hygiene patterns were unaltered, except that interproximal plaque was allowed to accumulate in an interproximal space adjacent to one of the troughs. The appliance was removed once per day to facilitate cleansing of the lower lingual aspects of the natural dentition. The acid/anion profiles of the appliance samples were similar to plaque of equal maturity from the adjacent interproximal site. Qualitatively the microbiological species recovered from the natural and appliance plaque were similar and within the normal range for 7- to 9-day natural plaque. When the composition of appliance and natural plaque was compared some quantitative differences were found. Thus it would seem that the previously reported de- and remineralisation investigations using this appliance were carried out under the influence of an ecosystem similar to that of an adjacent interproximal site of the natural dentition. In addition, this appliance should provide a means for the study of the microbiology and biochemistry of early enamel caries.

The study of enamel demineralisation and remineralisation in the natural oral environment with its complex biological and biochemical interactions has been the goal of many workers.

In a previous text [Creanor et al., 1986] a new enamel section-carrying intra-oral appliance was described, in which successful de- and remineralisation was achieved. It was important to establish if these de- and remineralisation studies had occurred under the influence of an ecosystem similar to that of the natural dentition. Therefore, it was decided to compare the microbiological and acid/anion profiles of the plaque-like material which accumulated at

the 'contact point' of the sections on the appliance with those of plaque taken from an adjacent interproximal area of the volunteer's natural dentition. The two methods used to test the composition of the deposits which formed on the appliance were acid/anion profile analysis and microbiological assessment. These tests were chosen because the changes in organic acid levels of fasted plaque after exposure to sucrose have been investigated thoroughly [Distler and Kroncke, 1983; Geddes, 1975; Geddes and Weetman, 1983; Gilmour et al., 1976] and the bacteria normally present in 7- to 9-day-old dental plaque have been well characterised [Ritz, 1967].

Materials and Methods

General

The in situ caries appliance described in a previous article [Creanor et al., 1986] was again employed. Enamel sections were cut from premolar teeth, extracted for orthodontic purposes, using a Leitz 1600 rotating annular diamond blade to an approximate thickness of 230 µm. They were then hand-ground to a final measured thickness of between 100 and 120 µm and coated using a proprietary nail varnish on all cut aspects, leaving only the natural enamel surface exposed. Sections were subsequently mounted on the base of the trough of the appliance of 1 volunteer with their non-varnished surfaces opposed, thus mimicking a 'contact point' as shown in figure 1. The volunteer was instructed to wear the appliance for 7 days, during which the individual's normal dietary pattern was maintained and a non-fluoridated dentifrice used. The appliance was removed once per day to facilitate cleansing of the lower lingual aspects of the natural dentition by brushing and flossing, apart from one interproximal area in the 76| region; thus plaque accumulation on the volunteer's dentition was kept to a minimum, although that developing in the experimental trough was left undisturbed. The procedure was repeated on several occasions as detailed below.

Plaque Sampling

At the end of the 7- day period the volunteer was instructed to refrain from both late night snacks the day before and from breakfast on the morning of appliance removal. A sample of the 7- day-old plaque was taken for acid/anion profile estimations from the exposed enamel surface of one of the sections (fig. 1) using a Gillette No. 11 scalpel blade and × 10

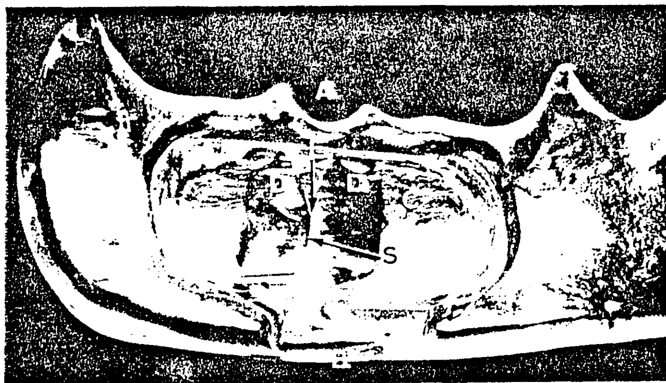

Fig. 1. One side of the appliance showing the entrance (A) and the exit (B) to the trough (C) situated on the fitting surface. Enamel sections (D) are mounted in a 'contact point' situation (E). Plaque samples (S) were taken from the exposed natural surfaces of the sections (D).

magnification, to minimise contamination of the specimen. A sample was then taken from the natural interproximal region in the $\overline{76|}$ area, also for isotachophoresis. Both samples were placed immediately onto the tops of two plastic containers with some moist cotton wool in the base and stored at 4 °C. Within 10 min the sample was removed and placed into a preweighed plastic vial and reweighed ensuring at least 1 mg wet weight of sample. Not later than 30 min from the time of sampling, the plaque was mixed with the leading electrolyte (vide infra) at 4 °C, centrifuged at 20,000 *g* for 15 min and stored at −20 °C until analysed [Geddes and Weetman, 1983]. The appliance was reinserted immediately and the volunteer rinsed with 10 ml of a 10% sucrose solution for 30 s. After a further 4 min the appliance was removed and two additional samples were taken from each of the natural and appliance sites. One pair of samples were transported to the laboratory for isotachophoresis as described earlier, while each of the second set of samples was placed into 1 ml of sterile anaerobic blood broth (Gibco-Europe, Paisley) and taken immediately to the microbiology laboratory.

Acid Anion Estimations

Acid anions were analysed by isotachophoresis using an LKB 2127 Tachophor (LKB Instruments, Bromma, Sweden) fitted with conductivity and ultraviolet detection systems. The separation took place in a 610-mm Teflon capillary tube of 0.5 mm diameter maintained at a constant temperature of 12 °C. The detection current was 50 μA.

The leading electrolyte was 5 m*M* hydrochloric acid adjusted to pH 4.2 by the addition of 6-amino-n-hexanoic acid, which acted as the buffering counter ion. Hydroxypropylmethylcellulose (0.2% w/v) was added to the leading electrolyte to sharpen zone boundaries by reducing electro-endosmosis. The terminating electrolyte was 4 m*M* n-octanoic acid adjusted to pH 5.5 by the addition of 2-amino-2-(hydroxy-methyl)propane-1,3-diol(Tris). All chemicals were obtained from BDH Chemicals Ltd., Poole, England, except hydroxypropylmethylcellulose, which was obtained from Sigma Chemical Company Ltd., Poole, England. Double-distilled deionized water was used to prepare the electrolytes.

Quantitative data were obtained by reference to standard runs of lactic, acetic, propionic, succinic, pyruvic, formic and phosphoric acids. Finally, statistical analysis was performed using a t test.

Microbiology

Plaque samples were dispersed by vortex mixing for 60 s, followed by sonication for 15 s at a setting of 1.5 (Ultrasonic Sonicator, Heat Systems Ultrasonics, Plainview, N.Y.). Serial tenfold dilutions of plaque samples were immediately made from neat to 10^{-8} in anaerobic blood broth (Gibco-Europe). A standard inoculum (0.1 ml) of each dilution was inoculated onto each of the following media: Trypticase Soy Blood Agar supplemented with vitamin K and haemin (Gibco-Europe), Mitis Salivarius Agar supplemented with 20% sucrose and 20 units bacitracin per 100 ml (Difco, Surrey), and Rogosa's Agar (Difco, Surrey). The inoculum was spread over the surface of the agar evenly using a right-angled sterile glass rod and the plates incubated under anaerobic conditions for 4 days at 37 °C. On average 15 min elapsed from the time of sampling to incubation of the plaque cultures. Plates with easily counted numbers of colonies were selected and the total bacterial, *Lactobacillus* spp. and *Streptococcus mutans* counts were calculated. All the colonies from the blood agar plate which was selected for counting and three colonies from each of the selective media plates were subcultured onto blood agar and incubated until growth occurred. The isolates were identified using standard technique, i.e. morphology, growth conditions and biochemical tests. Streptococci were identified using Appareils et Procédés d'Identification (A.P.I. Strep., Basingstoke, Hampshire), anaerobic rods by Minitek (Becton-Dickinson, Oxford) and gram-positive rods by a combination of Minitek and acid end-product results. Finally, the percentage of each species in the original plaque samples was calculated.

Results

Acid Anion Profiles

The results for the lactate, acetate and propionate profiles for both the appliance and natural plaques are listed in tables I, II and figure 2. Statistical analysis showed that the lactic acid levels were significantly different after the sucrose rinse for both the natural ($p < 0.001$) and appliance ($p < 0.05$) plaques. The acetate and propi-

Table I. Acid/anion concentrations (nmol/mg wet weight) from 1-week-old appliance plaque before and after a 10% sucrose rinse

Appliance	Lactate		Acetate		Propionate	
	before	after	before	after	before	after
Sample 1	2.8	53.0	15.5	16.9	3.0	4.4
Sample 2	13.7	74.0	27.2	26.5	13.1	9.0
Sample 3	3.1	48.0	23.4	17.2	1.9	2.8
Sample 4	1.5	12.8	22.3	19.8	5.2	3.8
Sample 5	8.0	21.3	16.1	19.9	2.1	1.9
Sample 6	1.4	12.7	22.2	19.9	5.2	4.0
Mean	5.1	37.0	21.1	20.0	5.1	4.3
SD	±4.5	±23.0	±4.1	±3.2	±3.8	±2.3

Table II. Acid/anion concentrations (nmol/mg wet weight) from 1-week-old natural plaque before and after a 10% sucrose rinse

Natural	Lactate		Acetate		Propionate	
	before	after	before	after	before	after
Sample 1	2.9	48.6	42.9	48.1	5.2	4.8
Sample 2	2.7	31.2	24.1	25.8	1.6	3.6
Sample 3	4.0	23.3	28.9	34.0	5.5	6.9
Sample 4	4.2	28.3	8.6	12.7	1.6	3.8
Sample 5	9.4	23.0	33.1	32.9	6.9	3.3
Sample 6	4.2	16.6	8.2	6.8	1.4	2.1
Mean	4.6	28.5	24.3	26.7	3.7	4.1
SD	±2.2	±10.1	±12.6	±13.8	±2.2	±1.5

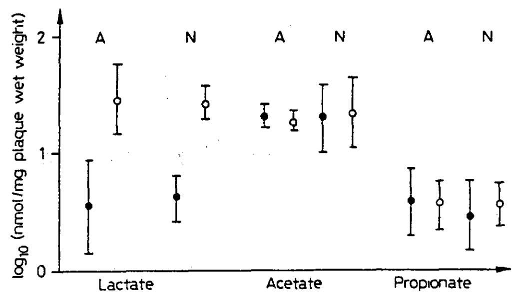

Fig. 2. Logarithm to the base 10 (±1 SD) of various acid/anion concentrations (nmol/mg wet weight plaque) from the appliance (A) and natural (N) plaque samples, before (●) and after (○) a 10% sucrose rinse.

Table III. The predominant cultivable flora from 1-week-old 'appliance' and natural plaque

	Mean % of total cultivable flora		Range, %	
	appliance	natural	appliance	natural
Streptococcal spp.	48.5	43.3	47.2–53.2	6.2–80.1
Gram-positive rods (predominantly *Actinomyces*)	9.0	27.0	10.9–12.4	2.2–51.9
Gram-negative rods (predominantly *Bacteroides*)	8.7	5.7	7.0–10.0	0.1–11.3
Veillonella spp.	15.4	7.9	12.5–21.8	4.3–11.4
Fusobacteria spp.	5.1	6.3	0.9–8.0	2.2–10.1
Capnocytophaga spp.	6.0	3.3	5.3–6.4	1.4–5.1
Individual species				
Streptococcus sanguis type 1	6.2	0.0	0.0–18.6	0–0
Streptococcus sanguis type 2	25.6	14.6	21.3–29.0	2.5–25.7
Streptococcus mitior	5.3	0.0	0.0–12.0	0–2.2
Actinomyces odontolyticus	3.5	0.8	0.0–10.6	0–1.5
Actinomyces naeslundii/viscosus	0.7	22.1	0.0–1.3	0.0–46.8
Bacteroides intermedius	2.3	0.0	0.3–4.0	0–0
Anaerobic streptococci	10.8	28.8	0.0–18.2	3.7–52.3

onate levels were unaffected by sucrose rinsing. There were no significant differences in the levels of all the acids tested between the natural and appliance plaque either before or after sucrose rinse.

Microbiology

The microbial composition of the plaque which formed on the enamel sections mounted on the appliance during each of the experiments was relatively consistent compared with the natural plaque which showed more variation (table III). Microorganisms not normally present in dental plaque, e.g. *Candida*, coloforms or staphylococci were never isolated. In addition *S. mutans* was absent from both blood agar and Mitis Salivarius Bacitracin agar cultures, while lactobacilli were isolated on two occasions, once each from appliance and natural plaque samples.

Discussion

The acid/anion results from this study are similar to those reported by Geddes [1975], Vratsanos et al. [1979] and Distler and Kroncke [1983], who have analysed pooled plaque from fasted subjects before and after sucrose rinsing. In the present study, like those cited above, it was found that the lactic acid exhibited a steep increase following a sucrose rinse. The concentrations of pyruvate, phosphate, formate and succinate both before and after sucrose rinse although not significantly different for both the plaque samples, were present in minor amounts and therefore details were not presented. Similar findings have been reported previously [Geddes, 1984], and therefore any major difference between the natural and appliance plaques would be more evident in the

changes in the levels of the lactic, acetic and propionic acids.

It is well known that the microbial composition of dental plaque varies considerably at different sites on the same tooth and at the same site on different teeth [Marsh and Martin, 1984]. In addition, since the composition of dental plaque fluctuates on the same tooth and site with time, the qualitative and quantitative variations which occurred in the appliance and natural plaque samples are regarded as acceptable. The composition of the deposits which formed on the enamel of the in situ applicance was within the range normally accepted for dental plaque collected 7–9 days after thorough tooth prophylaxis [Ritz, 1967], although this was less true for the natural plaque samples.

The pilot results of both the acid/anion profiles and the microbiological estimations would suggest that the deposits which accumulate on the exposed enamel surfaces mounted on this new intra-oral caries investigative device have both microbiological and biochemical properties similar to dental plaque.

Thus the successful creation of an accurate artificial oral stagnation area via this new in situ system should enable more realistic microbiological and biochemical studies of early enamel demineralisation than has hitherto been possible. Also, as a dynamic accumulation of plaque occurs on the specimens under study, the presence of plaque as a barrier to remineralisation may be more clearly understood. In addition, the opportunity also exists to employ this device for the monitoring of potential plaque-inhibiting agents.

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