LOW FLUORIDE CONCENTRATIONS: THEIR RELEVANCE TO THE INHIBITION OF DENTAL CARIES.

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Summary.

The beneficial effects of fluoride in the prevention of dental caries are well established. The mechanisms whereby fluoride exerts these effects continue to be investigated extensively, whilst methods of fluoride delivery remain the subject of intense scrutiny. The aim of this thesis was to investigate the effects of reduced concentrations of fluoride in dentifrices on the levels of fluoride in saliva, and to examine their potential role in the inhibition of artificial carious lesions, *in vitro*.

A preliminary experiment studied ambient salivary fluoride levels three times weekly, in twelve subjects using dentifrices with fluoride concentrations of 0, 500, 1,000 and 1,500 ppm F (as sodium fluoride) in random order, each for one month. The results showed a significant carry-over effect. Hence, a definitive experiment was designed which employed increasing concentrations of dentifrice fluoride. Here, 20 volunteers had fluoride levels measured in saliva three times weekly whilst using the aforementioned concentrations of fluoride dentifrices for one month each. In addition, ten of these volunteers had fluoride levels assayed in plasma and plaque once per week. These data provided a range of ambient salivary fluoride values of from 0.01-0.04 ppm.

As a result of the above, this fluoride concentration range was used in a subsequent *in vitro* pH-cycling experiment, which employed a human enamel single section technique utilising 100-150μm longitudinal tooth sections. Subsequent de- and remineralisation was assessed using microradiography and microdensitometry. The ΔZ results indicated there were significant differences in the levels of demineralisation in lesions cycled through solutions containing this low range of fluoride concentrations.

Subsequent experiments were performed to examine patterns of fluoride clearance from plasma and parotid duct saliva and the role of oral mucosa was investigated in prolonging the intra-oral clearance of fluoride following its topical application. These experiments demonstrated that the oral mucosa plays a substantial role in prolonging the clearance of salivary fluoride by acting as a reservoir, and thus influences indirectly the ambient salivary fluoride concentration.

The results of these experiments indicate that, lower fluoride-containing dentifrices reduce the ambient level of fluoride in whole saliva, and that *in vitro,* these lower levels of fluoride are associated with increased demineralisation of artificial carious lesions. Moreover, the oral mucosa is a site of fluoride retention from dentifrices and mouthrinses, and contributes to the ambient salivary fluoride levels. Thus it may be partly responsible for the prolonged nature of oral fluoride clearance and it would seem logical to conclude that further clinical and laboratory research on both the formulations and delivery of lower fluoride dentifrices is required.

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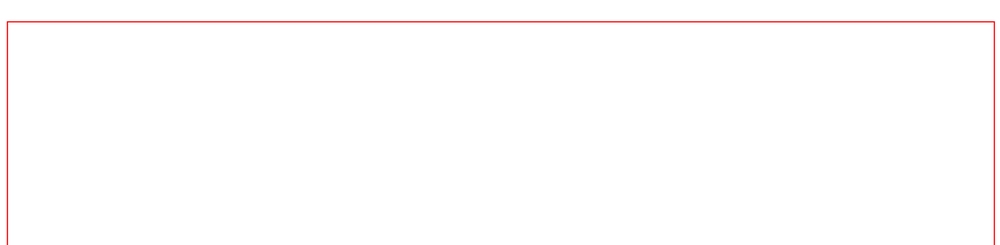
Declaration.

This thesis is the original work of the author.



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Chapter 1. Introduction.

1.1 Introduction.

Dental caries is widely recognised as the most prevalent disease of modern times. Its close association with the consumption of fermentable carbohydrate can be traced historically and geographically over the last four centuries, in a relationship which persists into the present day (Gustafsson *et al.*1954; Jenkins,1966). The introduction of fluoride, in a great variety of forms and regimes, has had a profound impact on the progression of this disease, and is further explored in this thesis. The changing patterns of caries prevalence and the clinical implications of this disease are the subject of this introductory chapter.

1.2 Dental Caries.

1.2.1 Epidemiology of Dental Caries.

It has been widely accepted over the last ten years, that caries prevalence amongst the young of most Western countries has been declining steadily (Renson, 1986). This trend has been most marked in the 5-year-old age groups. In England and Wales, for example, caries experience in this cohort fell by 50 per cent, by about 40 per cent in 12-year-olds and by about one third in 15-year-olds, between 1973 and 1983 (Todd & Dodd, 1983). Similarly, surveys carried out in Sweden (Stecksen-Blicks, Holm & Mayanagi, 1989) have indicated a decrease in deciduous, decayed, missing and filled teeth (dmft) for 4-year-olds, from 5.3 to 1.6 over the period 1967 to 1987. The percentage of this age group which was caries-free increased from 17% to 58% over the same time-frame.

In the United States, deciduous, decayed, missing and filled surfaces (dmfs) levels for 5-year-olds fell from 0.15 in the early 1970's to 0.07 in the late 1980's, and figures for 12 and 15-year-olds showed an almost identical percentage reduction (Renson, 1986). The National

Institute of Dental Research (NIDR) survey of caries in children, completed in 1987, documented a mean overall reduction for all age groups (5 years to 17 years) of 36% for permanent, decayed, missing and filled surfaces (DMFS), from 4.77 to 3.07, and nearly 50% of children in 1987 were judged clinically caries-free in their permanent dentitions (Brunelle, 1990).

The above figures would seem to paint an optimistic picture. Unfortunately, they tell only a part of the story. In many of the most densely populated regions of the world, caries prevalence is on the increase, and even in those areas where a degree of complacency has been creeping in, early indications of a small but significant increase are being documented. Countries such as Nigeria, Thailand, Chile, Mexico, India and South Africa have all, according to the World Health Organisation (W.H.O.) Data Bank (1989), shown alarming rises in caries prevalence amongst 12-year-olds over the last few years. The major contributory factor in this increase in the so-called "developing" countries, is the greater availability and consumption of refined, fermentable carbohydrate in diets which, until recently, were based on traditional foodstuffs. In most of these countries access to dental care is limited, and the pattern of decay followed by restorative treatment which is predominant in the West, is unlikely to be replicated. This may provide a useful opportunity to monitor the impact of a less interventionist, more preventive-orientated programme of dental care.

In addition, even in "the West", there is mounting evidence that the caries decline documented in the 1980's may be over. For example, the mean dmft for 5 year-olds in Scotland has increased from 2.73 to 2.88 over the years 1987 to 1993 (Scottish Health Boards Dental Epidemiology Programme, 1987/88 and 1991/92) but the reasons for such increasing disease prevalence in countries like the U.K. are elusive (Mandel, 1985). Whilst the advent of fluoridated dentifrices, and their eventual dominance of the toothpaste market, has been credited largely with the reduction in caries rates noted over the last decade,

children who are the subject of the above surveys have utilised fluoridated dentifrices throughout their lives. It may be there is some form of saturation effect, which is responsible for the plateau, (and in some areas an actual increase), in the number of tooth surfaces affected by decay.

In addition, the concept of a "high risk" section of the population is receiving considerable attention. Data from many countries show that disease levels in any given age-group are by no means distributed normally. In the U.K. for example, in 1983, 15-year-olds in Scotland and Northern Ireland had a Permanent, decayed, missing and filled teeth (DMFT) of 8.4 and 9.2 respectively, compared with 5.6 in England (Office of Population Censuses and Surveys,1986). An Australian survey of 12-year-olds (Spencer,1986) demonstrated this skewed distribution well, with the average DMFT dropping from about 9.0 to 3.0 over the period 1954 to 1982, whilst a small though significant percentage of the study population scored a DMFT of 9.0-18.0. Recent results from the S.H.B.D.E.P. studies (Dental Health Services Research Unit.,1991) have confirmed an increase in the percentage of 5 year-olds who are caries-free, but a concomitant increase in the mean dmft for the same population. This can only indicate a further rise in the disease prevalence in a specific section of the sample population. Indeed, in many countries 60 - 70 % of the disease is found in only 20% of the population (Bohannan *et al.*1985).

It seems reasonable to consider this 20% as a "high risk" group and the underlying factors which render these individuals more susceptible to disease are the object of considerable interest. A general perception of dental health as a low priority, relatively poor provision of dental services, an unwillingness to alter dietary habits, and reduced frequency of usage of fluoridated agents would all seem to be significant factors.

In general, these findings indicate there is no room for complacency if the recommendations of the W.H.O (World Health Organisation/Fédération Dentaire Internationale,1982) that

50% of 5-year-olds, world-wide, should be caries-free by the year 2000, are to be even approached.

1.2.2 Dental Enamel : Normal Structure.

Enamel is the ectodermally-derived tissue covering the anatomical crown of the tooth and is the most highly mineralised tissue of the body, consisting of 96% mineral and 4% organic material and water (all by weight). The inorganic content of enamel consists of a crystalline calcium phosphate known as hydroxyapatite or HAP ($Ca_{10}(PO_4)_6(OH)_2$), which is also found in bone, dentine and cementum. The susceptibility of these hydroxyapatite crystals to dissolution by acid provides the chemical basis for the carious process.

Because of its highly mineralised nature, the structure of enamel is extremely difficult to study. Enamel prepared in ground section and examined under the light microscope reveals a structure composed primarily of elongated units termed "rods". When these rods are magnified further and examined at electron microscope levels, they are seen to be cylinder-like and consisting of crystals with their long axis, for the most part, running parallel to the long axis of the rod. Crystals more distant from the central axis flare laterally to an increasing degree as they approach the rod periphery. The boundary where the crystals of the rod meet those of the inter-rod region, which run at a different orientation, is known as the rod sheath. The cross-sectional outline of these two related components has been compared to the shape of a keyhole, and the basic unit of enamel is most appropriately described as a cylindrically shaped rod that has a special relationship to the inter-rod region directly cervical to it. This configuration of enamel crystals is a direct result of the progression of the secretory ameloblasts and their attendant Tomes' processes.

Enamel rods have an average width of 5μm, but vary in size and morphology throughout the thickness of enamel. In the enamel immediately adjacent to dentine there is no rod structure, and towards the enamel surface the rods are either irregular or absent. In between, as they

traverse the enamel, the rods gradually increase slightly in diameter.

The organic content of fully mature enamel consists largely of a group of proteins known as enamelins, and these are found predominantly in the inter-rod regions and the rod sheaths. These areas contain more enamel protein than other regions because crystals meeting at different angles cannot be packed as tightly together. The consistent arrangement of rod sheaths with their greater protein content accounts for the "fish-scale" appearance of enamel matrix seen in sections of demineralised developing enamel, and in etched ground sections.

The significance of this ultra-structural arrangement of enamel for the initiation and progression of dental caries is discussed in the next section.

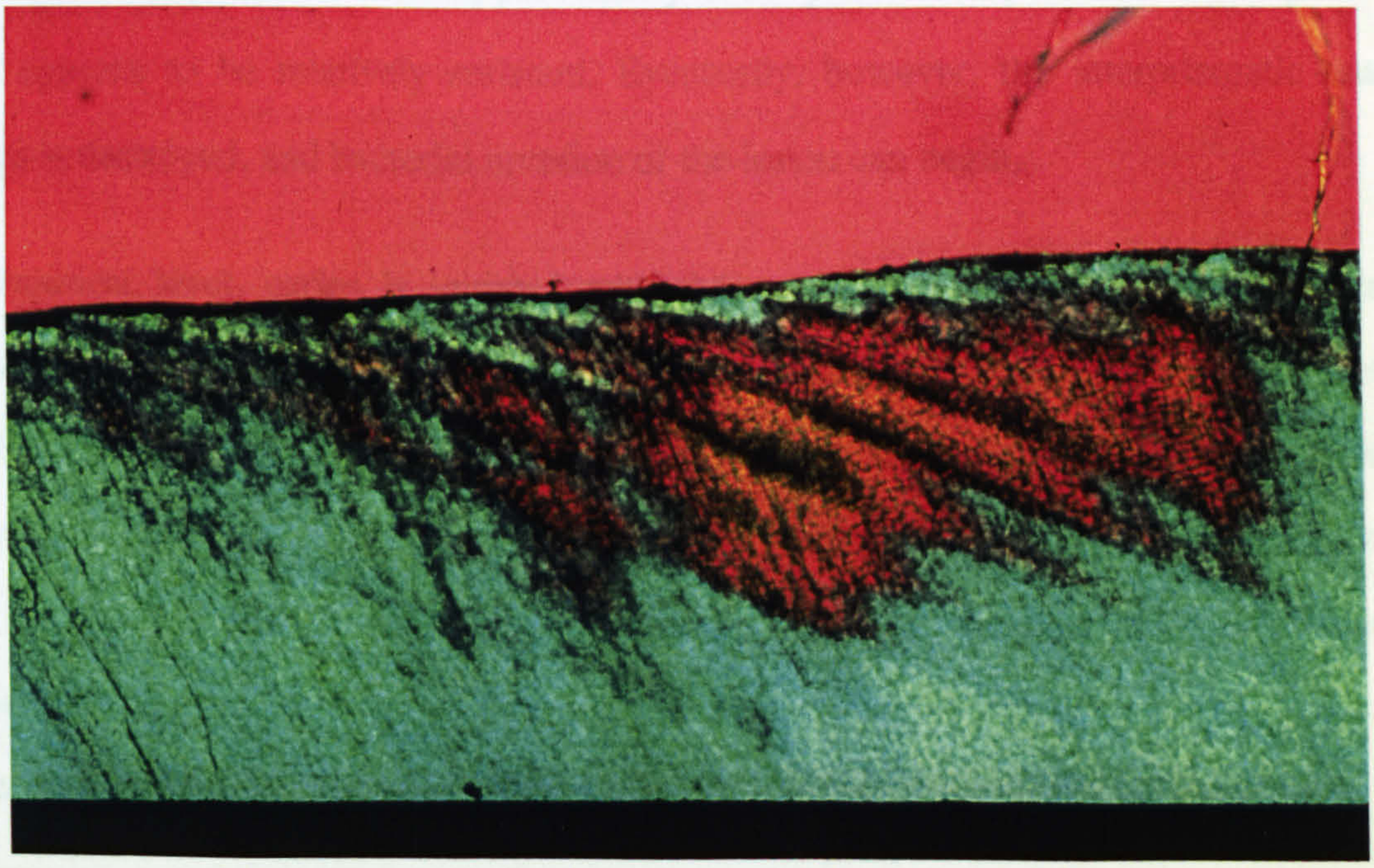
1.2.3 Initiation of the Carious Lesion.

It is now generally recognised that dental caries is an infectious disease which causes the localised destruction of the coronal and radicular dental hard tissues, induced mainly by acids produced in dental plaque (Nikiforuk, 1985; Thylstrup & Fejerskov, 1985). Although the carious process occurs in all three of the dental hard tissues, it is the particular pattern of enamel demineralisation and remineralisation which is of interest to the work reported in this thesis, and which will therefore be discussed here.

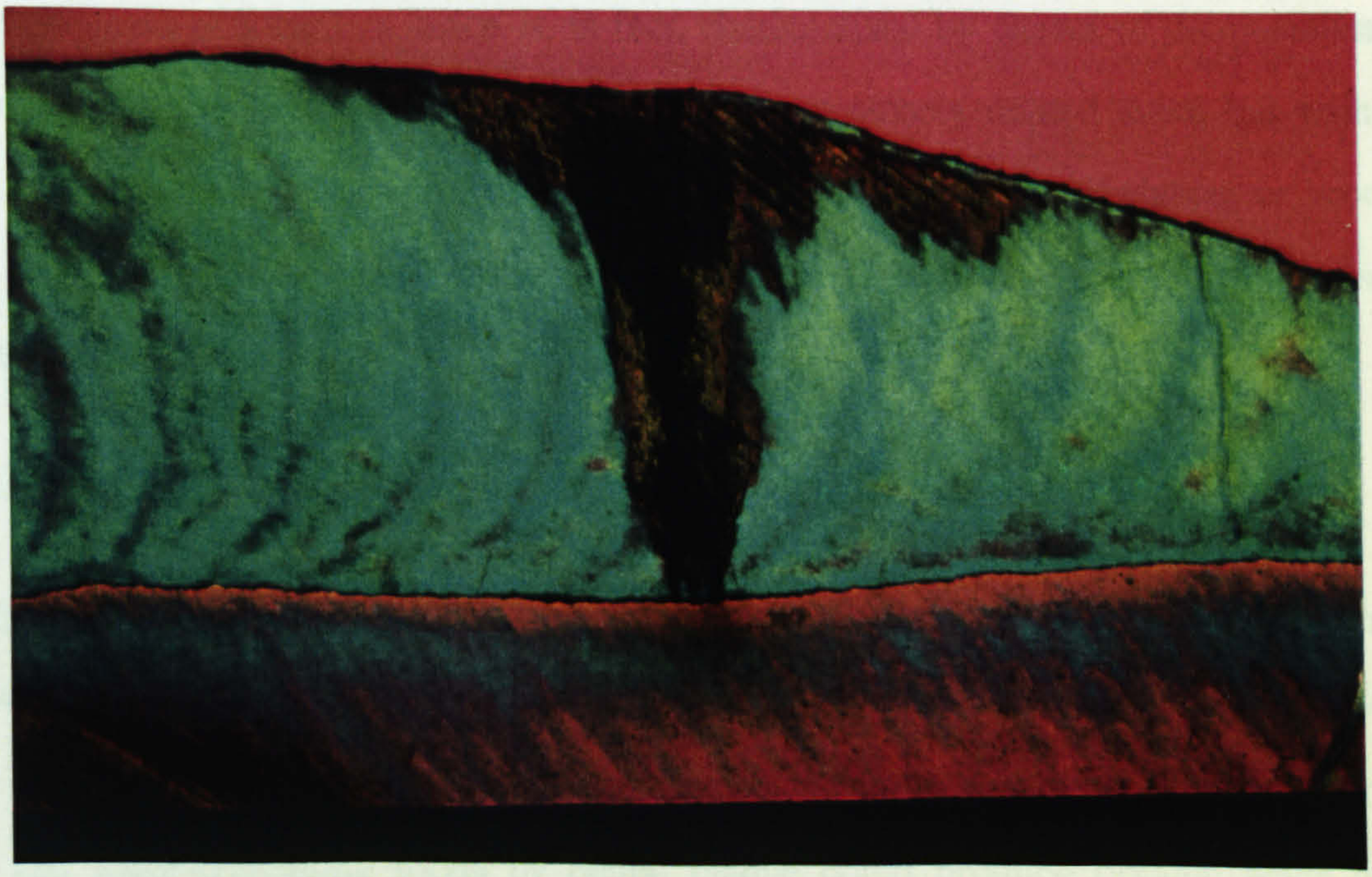
The earliest clinically indication of enamel caries is the appearance of the "white-spot" lesion which results from a change in the optical properties of the superficial layers of mineral. It is now well-recognised that this incipient lesion in enamel consists of a relatively intact surface layer, overlying a demineralised sub-surface zone (Darling, 1958; Silverstone, Hicks & Featherstone, 1988). The use of polarising microscopy has identified four different regions within the early enamel lesion (Fig 1.1). Moving from the surface inwards, these are the surface zone, the lesion body, the dark zone and the translucent zone. The surface zone

Figure 1.1 **Photomicrographs of longitudinal ground sections through artificial carious lesions viewed with polarised light: (a) an early lesion and (b) a lesion extending to the amelo-dentinal junction.**

(a).



(b).



shows less mineral destruction than the lesion body (Frank, 1989) and where the surface layer is prismatic, a distinctive broadening of the prism sheath areas occurs. These enlarged sheath areas can be followed from the enamel surface to the body of the lesion and would seem to provide minute pathways from the surface, where any dental plaque would be located, to the sub-surface lesion. A progressive widening of the sheath areas is followed by dissolution of the prismatic enamel, particularly in the prism cores, whilst the interprismatic enamel appears to be relatively resistant. Eventually, however, the interprismatic enamel structure is destroyed, and bacterial invasion of the lesion can begin.

At a chemical level, caries is initiated with the production of organic acids by plaque bacteria, causing the release of calcium and phosphate ions from the partial dissolution of the enamel apatite crystals. Depending on the local pH, and on the ions present in the immediate vicinity, different types of calcium phosphates and other calcium compounds can form. It should be emphasised, however, that this is not a static situation, and these calcium compounds can transform into other phases, as dictated by changes in local physico-chemical conditions.

The development of a carious lesion is, in fact, characterised by phases of demineralisation and remineralisation, often occurring simultaneously at closely related sites, but with the overall balance of mineral flux favouring a net demineralisation. Any substance, or process, which has the potential to influence either of these mineral changes, may have some cariostatic effect.

1.2.4 The Role of Micro-organisms in Dental Caries.

As long ago as 1697, work by van Leeuwenhoek (Mellville & Russell, 1983) determined that the oral cavity, and the teeth in particular, could support bacterial colonies. Miller (1883) made significant advances in caries aetiology when he reported that fermentation of starch and sugar-containing foods by dental plaque could produce organic acids which

would cause dissolution of enamel and dentine.

It was not until the study by Orland *et al.,* (1954) that bacteria were established as an essential factor in the initiation and progression of dental caries *in vivo*. These workers developed a technique of raising germ-free (gnotobiotic) rats, delivered by Caesarean section and fed on a sterile diet. These gnotobiotic rats were compared with a control group with a normal oral flora, fed on the same diet. At the end of the experiment examination of the molar teeth showed carious lesions in the control group, but no lesions in the germ-free rats. This was the first study to demonstrate that caries could not be initiated *in vivo* in the absence of bacteria.

In order for the oral flora to remain viable there must be a renewable supply of appropriate bacterial substrate. The presence of this substrate *per se*, constitutes a primary factor in the aetiology of caries initiation and progression. The type of substrate, and its rate of renewal are regarded as secondary factors, in that they modify the primary factors for caries initiation and progression (tooth enamel, oral flora and bacterial substrate), but are not essential elements of the disease aetiology.

A classic investigation by Gustafsson *et al.* (1954), known as the "Vipeholm Study" was undertaken in Sweden to determine the effect of variable carbohydrate levels on the incidence of caries in 436 institutionalised individuals over a five year period. All participants were placed on a basic diet, judged adequate in all respects except calorific content. This was increased by adjusting the fat or sugar content, both in total quantity and in frequency of intake. Four different dietary groups were developed, namely:

1. Basic diet with added fat (The control group).

2. Basic diet with added sugar (300g) in solution at mealtimes.

3. Basic diet with added sugar (50g) in bread, initially with afternoon coffee (years 1 and 2),

then with all meals i.e. four times per day (years 3 and 4)

4. Basic diet with sugar as confectionery, consumed between meals. This group was subdivided into those eating chocolate, those eating caramels, and those eating either eight or twenty four toffees per day.

The results of these studies were summarised by Nikiforuk (1985), who concluded that caries levels increased with raised dietary sugar intake and, significantly, caries levels were increased further when sugar was consumed between meals, was retained at the tooth surface, or had a reduced rate of clearance. However, caries still occurred in subjects maintaining a low level of refined carbohydrate in their diet.

Additional well-known studies investigating the role of bacterial substrate in human caries development were those undertaken at Hopewood House, Bowral, Australia in 1952-1961, which examined a group of children following a specific dietary regime. Harris (1963) described this diet as "notable for the almost complete absence of refined carbohydrate and the presence of minimal animal protein ". The studies of Sullivan & Harris (1958) and Harris (1963) compared the dental health of the Hopewood House children to data from a state school survey published by Barnard in 1956, and reported the Hopewood House subjects had significantly less caries. Whilst the results of these studies may be, in part, due to the timing of food intake, the authors concluded that the major factor was the difference in the amount of bacterial substrate available to be metabolised by plaque bacteria to produce organic acids.

More recently, however, Bibby (1990) has suggested that the role of sucrose in caries aetiology has been over-played. He argues that the increased sucrose usage by primitive peoples is often associated with an increase in the use of wheat flour, that the rationing of sugar during the First and Second World Wars was also accompanied by a rationing of flour, and that the amount of sugar consumed by the subjects in the Vipeholm study did not

parallel the caries incidence observed. In addition, a study by Woodward & Walker (1994) analysed data for sugar consumption and caries prevalence in 90 countries, and were unable to demonstrate a sugar:caries relationship from the 29 industrialised countries' data. They proposed that other factors, such as exposure to fluoride, should be taken into account when recommendations for caries control are being made on a population basis. These findings and conclusions further emphasise the multi-factorial nature of the caries disease process.

1.2.5 Progression of the Carious Enamel Lesion.

The spread of a carious lesion is determined basically by the direction of the rods and crystals in the immediate vicinity, and the distribution of the overlying plaque deposits. In some cases, the extension of the lesion may reach the amelo-dentinal junction without any macroscopically visible breakdown or cavity formation (Pitts & Rimmer, 1992), whilst in other instances, an extensive degree of subsurface demineralisation, combined with mechanical damage of the outer surface layer, may create a cavity extending partly into the enamel but without direct communication into the dentine (Wenzel, Larsen & Fejerskov, 1991). In addition, the higher organic component of the Striae of Retzius results in the preferential diffusion of organic acid down these channels, providing an initial pathway for the demineralising front to advance towards the amelo-dentinal junction.
When a carious lesion reaches the amelo-dentinal junction, the then highly porous enamel allows for a further diffusion of acids into the dentine. An immediate demineralisation is seen at the apex of the advancing enamel lesion, which then spreads peripherally along the amelo-dentinal junction. In dentinal tubules corresponding to the demineralisation area, as well as those immediately peripheral to it, a tubular sclerosis is usually evident.

When the enamel lesion finally becomes so demineralised throughout the enamel thickness that the tissues break apart, a carious cavity filled with plaque micro-organisms develops. The microbial mass which then also invades the dentine, consists of a mixed flora which produces a range of hydrolytic enzymes with the potential for the destruction of the organic matrix of dentine. In some areas, demineralisation and proteolysis occur so rapidly that

several groups of affected tubules coalesce to form liquefaction foci. From this stage, physical breakdown of the dentine, and undermining of the overlying enamel allow for rapid extension of the lesion which represents an urgent requirement for clinical intervention if viability of the pulp is not to be threatened by direct bacterial invasion.

1.2.6 Clinical Implications of Tooth Loss.

Whilst it is only in the rarest of circumstances that dental caries can produce life-threatening sequelae, the sheer morbidity of the disease, and the difficulties associated with its long-term treatment, ensure its classification as a significant clinical entity. The restoration of simple one or two surface cavities is relatively straightforward, expedited by recent improvements in dental materials, but the treatment of multi-surface lesions, endodontically-involved teeth, and the replacement of extracted teeth with fixed or removable prostheses are both technically and clinically demanding. It is well established that once a tooth surface has undergone initial restoration, it is inevitable that, at some unspecified point in the future, it will require a subsequent and probably more extensive restoration, due mainly to the breakdown of the marginal areas between the enamel and the filling material. This occurs as bacteria colonise this sheltered niche, which even the most clinically "perfect" marginal adaptation provides. This restoration/replacement cycle provides one of the major motivations for preventing the occurrence and progression of incipient lesions before they reach an extent which renders restorative intervention necessary. The alternative progression through simple fillings, jacket crowns, endodontics, post crowns, bridges and eventually to dentures is extremely time-consuming, expensive and ultimately can never match the levels of aesthetics and function provided by a natural and healthy dentition.

In areas where the provision of dental services is in a rudimentary state, or where the uptake of services is low, the consequences of an "extraction only" philosophy are seen clearly. Mesial drifting, over-eruption of unopposed teeth, increased wear or attrition of the

remaining dentition and difficulty in maintaining oral hygiene, all add to the complexity of providing satisfactory treatment and increase the propensity to periodontal disease.

The combined public and private outlay on dental care outside hospitals is currently running at over £1500 million a year in the U.K. (Taylor,1991), half being spent on restoring teeth attacked by dental caries and nearly £70 million on the provision of dentures to replace extracted teeth. It has been reported (Office of Health Economics,1969; Department of Health, 1991) that in terms of a single specific illness or disease, the cost of dentistry is second only to the cost of mental illness, and is greater than the direct National Health Service expenditure on all types of cancer, or on the treatment of heart disease.

Clearly, greater efforts towards public health education, provision of preventive-orientated dental health services, and continuing scientific and clinical research into the causative factors and potential treatments of early dental disease are prerequisites, if this situation is to be fundamentally improved.

1.3 Fluoride.

1.3.1 Introduction.

Fluoride has, for the last fifty years, been a cornerstone of the endeavours to prevent dental decay. Chemically fluorine is classed as a halogen, and is the most electronegative of all the elements. Through this strong reactivity, it forms fluoride salts with almost all metals. Although there are many aggressive forms of fluoride such as hydrofluoric acid, the most abundant solid forms (calcium fluoride and fluorapatite) are very poorly soluble in water, and entirely harmless.

Classically, the clinical data on the effect of water-borne fluoride have served as the most important information on the caries-reducing properties of small amounts of fluoride in the oral cavity. Current opinion is that, although it is likely that there is some pre-eruptive effect

of ingested fluoride, this is only of borderline significance relative to the post-eruptive effect produced by topical application of even very low concentrations e.g. 1 ppm. (Featherstone *et al.*1990).

1.3.2 Fluoride : Role in Caries Prevention.

An extensive range of fluoride vehicles has been studied over the last 20 years, including milk, salt and vitamin supplements. The most intensive efforts, however, have been reserved for water fluoridation and topical fluoride agents such as dentifrices, rinses and gels. Evaluation of these products has been undertaken traditionally either in human clinical trials, laboratory testing *in vitro* or, more recently, *in situ.*

As long ago as 1938, Miller , using a rat caries model, demonstrated that fluoride inhibited caries in erupted teeth. Klein (1946) and Backer-Dirks (1978) also showed that erupted teeth benefited from water fluoridation, and in 1971 Murray demonstrated a clear advantage to adults, in terms of a reduced DMFT, in addition to the improvement already documented in children.

More recently, comparisons between NaF* and $NaPO_3F$** dentifrices, and combinations of these two most widely utilised active ingredients, continue to reveal their significant anti-caries properties, with correctly formulated NaF in silica preparations proving superior (Stookey *et al.*1993; Stephen *et al.*1993; Conti *et al.*1993). It is generally accepted that the recent reductions in caries experience recorded in some Western countries can be attributed largely to the extensive usage of fluoridated dentifrices, to the extent that it is now considered unethical to use a placebo, or non-fluoridated dentifrice, as a control in a clinical trial.

Whilst past enthusiasm has been for the widespread use of topical agents formulated with relatively high concentrations of fluoride designed to be applied infrequently e.g. APF*** gel

* NaF = sodium fluoride
** $NaPO_3F$ = sodium monofluorophosphate
*** APF = acidulated phosphate fluoride.

(1.23% or 12,300ppm F), fluoride varnishes (Duraphat 2.26% or 22,600ppm F) etc., as a result of recent research, there appears to be tipping of the balance of current opinion in favour of frequent delivery of materials containing much lower levels e.g. 1000 ppm F dentifrices (0.1% F), applied twice daily (Mellberg,1991).

However recent research by Featherstone *et al.* (1990) has demonstrated a caries-inhibitory effect *in vitro*, of fluoride concentrations at the solid:liquid interface previously considered inconsequential i.e. 0.04 ppm F. This finding is significant for two main reasons - firstly these levels can be delivered safely to the oral environment via the public water supply, thus ensuring a continuous and widespread fluoride exposure and, secondly, such levels are incapable of producing the few deleterious side-effects of fluoride ingestion encountered with higher doses e.g. fluorosis, nausea, gastric erosions.

1.3.3 Mechanisms of Fluoride Action I : Introduction.

Historically, the cariostatic effect of fluoride in relation to the natural fluoride content of drinking water, was attributed to the systemic incorporation of fluoride into the tooth. It had already been discovered that acids caused dental decay, and it was widely accepted that the main mechanism of fluoride action was an effective reduction in the solubility of tooth enamel. Consequently, great emphasis was placed on the systemic administration of fluoride, with any topical application being considered of only secondary importance. This philosophy was given further weight by the 25-30% reduction in caries rates recorded in topical fluoride clinical trials, compared with the 50-60% reduction found in water fluoridation studies. The fact that the water fluoridation studies usually involved comparisons of groups which had been exposed to systemic fluoride for 10-15 years, as opposed to only 2-3 years of the topical agent studies, was largely overlooked.

The theory of reduced enamel solubility is based on the uptake of fluoride into the enamel lattice. The high electronegativity, low dissociation energy and small radius of the fluoride

ion, produces less strain in the x-ion site when it substitutes for the hydroxyl ion, and results in the reduced solubility of fluoridated apatite (FAP) over hydroxyapatite (HAP). Fluoride also helps eliminate impurities and defects in the crystal lattice by filling the voids left by missing hydroxyl ions. The hydrogen bond formed between the fluoride ion and the oxygen atom of the adjacent hydroxyl group is stronger than that of hydroxyl-to-hydroxyl bonds, and results in increased stability of the hydroxyl column and the HAP lattice (Nikiforuk, 1985).

Hallsworth (1984), have established that fluoride incorporated into enamel does not significantly alter its resistance to caries development. Nelson *et al.,* (1983) have shown that the dissolution rate of pressed synthetic hydroxyapatite was hardly influenced by the presence of 1000 ppm fluoride incorporated into the solid state. In addition, enamel which has erupted and matured in a fluoridated community contains only 500-1000 ppm fluoride (Weatherell, Naylor & Hallsworth, 1977), in contrast to a fully fluoridated apatite, which contains approximately 38000 ppm F. Apparently then, the reduced solubility conferred on HAP by the substitution of fluoride for hydroxide ions explains only partly, the anti-caries mechanism.

1.3.4 Mechanisms of Fluoride Action II : Effect of Fluoride on Enamel Demineralisation.

As early as 1959, it was reported by Manly & Harrington that the addition of fluoride to acetate buffers delayed the demineralisation of enamel. A comparison was made between the effect of pre-treating enamel with high levels of F, and the addition of fluoride at 1 ppm to the acetate buffers. The rate of demineralisation was slower in the fluoridated buffer system, in spite of the fact that the enamel contained only a fraction as much fluoride. The authors suggested that the precipitation of a fluoride-rich mineral layer at the enamel sites showing the greatest demineralisation was a possible explanation for their findings.

More recently, a carbonated apatite model has been used to represent enamel apatite, and the effect of fluoride on crystal growth was demonstrated directly (Nelson &

Featherstone, 1982). Here, dissolution of the carbonated apatite was measured in acetate buffers incorporating fluoride at different concentrations ranging from 0-50 ppm. Fluoride at 1ppm in the buffer reduced the dissolution rate of the carbonated apatite to that of hydroxyapatite and, at higher concentrations, further inhibited its dissolution.

It has also been postulated (Jeansonne & Feagin, 1979) that the effect of fluoride may be determined by the rate of transformation of the crystal surface into fluoroapatite (FAP). Under conditions where fluorapatite becomes supersaturated in solution, no sudden change in demineralisation rate was found. A rapid decrease in the demineralisation rate has been noted, however, at the saturation line of CaF_2, suggesting that the precipitation of this mineral may be a more effective inhibitor (ten Cate & Duijsters,1983). Fluoride-containing minerals, precipitating under acidic conditions, would seem to have a profound influence over enamel demineralisation.

1.3.5 Mechanisms of Fluoride Action III : Effect of Fluoride on Enamel Remineralisation.

Remineralisation of enamel lesions has been shown to occur by deposition of crystalline HAP which, *in vitro,* has demonstrated complete repair. *In vivo,* however, the process is considerably slower and the crystals never grow to the same dimensions. Morphologically there is no restriction to the precipitation of an HAP crystallite matrix, as the minerals of this group all have closely related crystalline structures. As a result of the lower solubility of FAP or FHAP(fluorohydroxapatite), the thermodynamic force for their precipitation is greater, relative to HAP. Fluoride would appear therefore, to both initiate and accelerate the precipitation of FAP and FHAP on an enamel matrix (Brown,1974; Amjad & Nancollas,1979).

When the impact of fluoride on remineralisation is being considered, a distinction should be made between the effects of high doses of fluoride applied for a short duration, and the

effects of much lower concentrations present in the remineralising fluid for extended periods. The topical application of fluoride gels and dentifrices results in large quantities of fluoride being absorbed into the lesion. Chemical analysis of white spots has revealed that these contain considerably higher concentrations of fluoride than the surrounding enamel (Dawes & Jenkins,1957; Hallsworth, Robinson & Weatherell,1971), demonstrating the increased affinity of demineralised areas for fluoride. This results in accelerated mineral precipitation in the outermost region of the lesion, reduces the diffusion within the lesion interior, and further delays the mineralisation of the lesion body. This phenomenon was also addressed by Silverstone *et al.* (1981) who conducted experiments in which lesions were remineralised in a series of solutions with different mineral ion concentrations. At low supersaturation (1 mM Ca, 0.6 Mm PO_4 at pH = 7) remineralisation took place throughout the lesion, whilst when using three times this concentration, remineralisation was limited to the surface layer.

When fluoride is present at continuously low concentrations, in conjunction with calcium and phosphate, it can diffuse into the lesion and precipitate as FAP or FHAP. *In vitro* investigations (ten Cate & Arends, 1977) have shown that low fluoride concentrations accelerate the initial mineral deposition in lesions and softened enamel. At a constant 1 ppm F in the remineralising solution, a two to three fold increase in the rate of precipitation was found.

1.3.6 Mechanisms of Fluoride Action IV: Effect of Fluoride on Bacterial Acid Production.

Fluoride has been known as an enzyme inhibitor for many years, and amongst the early attempts to explain its effect on caries, was the possibility that it inhibited acid production by plaque bacteria. Bibby & Van Kesteren (1940) showed that 1-2 ppm F had a detectable effect on acid production by pure cultures of various oral acidogenic bacteria, but 10 ppm was needed for decisive inhibition, and about 100 ppm for reducing growth.

The concentration of fluoride in plaque ranges from 13-55 mg F/g dry weight of plaque (Tatevossian,1980) and is influenced heavily by the fluoride intake of the individual. It is generally accepted that fluoride interacts with bacterial cells in a pH-dependent way, and this may be a function of the redistribution of fluoride species from tightly bound, to ionizable, to free, which is itself dependent on the local pH. Work by Hamilton, Boyar & Bowden (1985) demonstrated that fluoride inhibits bacterial glycolysis at the enolase step, by complexing magnesium, and thus removing this ion from the catalytic site. This results in a reduction in the supply of phosphoenolpyruvate which, in turn, inhibits the transport of bacterial sugars.

Although there is still considerable interest in the anti-bacterial role of fluoride, some more recent studies (Hamilton & Bowden,1988) on the *in vitro* growth and adaptation of bacteria in the presence of fluoride, have predicted that oral bacteria will grow and survive in the levels of fluoride which are routinely present in plaque.

1.3.7 Fluoride Intake.

There is currently no consensus on the levels of fluoride acceptable in various foods and beverages consumed on a daily basis. This is largely due to the discrepancy between the fluoride concentration and the bioavaliablity, which varies with such factors as the type of fluoride, the presence of complexing ions such as aluminium and calcium, and the physical quantity of food to be absorbed.

The greater part of the fluoride intake in man originates from food and water ingested each day. In general, the fluoride concentration in food reflects the fluoride concentration in the water where the food has been processed. Fish and shellfish are commonly quoted as containing relatively high concentrations of fluoride, but this is largely isolated in the bones and skin, which are often discarded in food preparation. Without these components, seafood generally contains less than 1 ppm F (Taves,1983).

Bottled mineral waters usually contain significantly higher concentrations of fluoride than tap water (MacFadyen, McNee & Weetman,1982; Levy, Toumba & Curzon,1993). Values of 1.5 - 7.0 ppm have been quoted, and largely reflect the fluoride content of the geological formation from which the water originates.

The other major dietary source of fluoride, particularly in the U.K., is tea (Nikiforuk, 1985). The commercial tea plant takes up fluoride from the soil and accumulates it in its leaves. Fluoride is released rapidly into tea infusions, mostly within 5 - 10 minutes, and the resultant fluoride concentration of the beverage can range from 0.5 - 4.0 ppm. If fluoridated water is used for brewing, the concentration will be correspondingly greater. In the U.K., where the consumption of tea is high, 4.4 - 10 mg of fluoride intake per day has been quoted from this source alone (Walters *et al.* 1983).

In infants, the daily dietary fluoride intake is largely determined by the feeding pattern i.e. breast feeding or bottle feeding. The fluoride concentration in breast milk is 0.006 - 0.012 ppm (0.3 - 0.6 μM), and appears to be independent of the water fluoride concentration consumed by the mother (Spak, Hardell & de Chateau,1983). This is in contrast to bottle or formula fed infants, who may consume approximately 200 times more fluoride in a fluoridated area than a corresponding breast-fed infant (Ekstrand, Hardell & Spak,1984). Chicken products are also a potential source of significant quantities of fluoride for infants. The relatively high levels (1 - 10.6 ppm F) are a result of the fluoride in bone fragments left during the manufacturing process.

A variety of other potential sources of fluoride exist, but most are of minor significance. Fluoride is found in organic combination in some drugs, for example, benzothiadizines used as diuretics, fluorosteroids and phenothiazines and fluorobutyrophenones used as tranquillisers. Fluoride is also present in some anaesthetic gases such as methoxyfluorane, enflurane and halothane, which can be metabolised to yield some ionic fluoride.

The major non-dietary source of fluoride, however, remains dental fluoride preparations. The dose normally recommended for children ranges from 0.25 - 1.0 mg daily, depending on age and the level of water fluoridation. After the application of topical fluoride products, a portion of the dose is inevitably, either inadvertently or intentionally swallowed (McCall *et al.*1983). This is of particular significance with the application of high concentration products such as APF gel (1.23 % F) and fluoride varnishes (Duraphat 2.26 % F) when administered to young children. Data (Barnhart *et al.*1974) show that the amount of fluoride ingested following a topical application is inversely related to age, and the small plasma volume of young children contributes to a significant elevation of plasma fluoride under these circumstances. The increased prevalence of fluorosis in some socio-economic groups, both in the U.K. and the U.S., may be related to the inadvertent absorption of fluoride from supposedly topical applications, and should provide a major incentive to rationalise the currently empirical nature of topical fluoride product concentrations.

1.3.8 Pharmacokinetics and Metabolism of Fluoride.

Fluoride absorption and distribution is governed by first-order kinetics (Ekstrand,1977) and a clear understanding of these is essential if its physiological, pathological and therapeutic mechanisms are to be interpreted. Blood plasma is considered to be the central compartment into which, and from which, fluoride must pass for its subsequent distribution and elimination. Three studies have indicated that fluoride is not bound by plasma proteins, or by any other plasma constituent (Chen *et al.*1956; Taves,1968; Ekstrand *et al.*1977). Therefore, it has been assumed that interstitial fluid and plasma fluoride concentrations are virtually identical. In studies on the soft-tissue distribution of fluoride (Whitford, Pashley & Reynolds,1979), the use of plasma fluoride concentrations rather than interstitial fluid has therefore been accepted.

The concentration of fluoride in plasma is a variable, being dependent on the level of intake

and several additional physiological factors (Whitford, 1989). Guy (1979) pointed out that, when plasma and water fluoride levels were expressed in terms of μmol/L and ppm respectively, the values were numerically identical. Thus, plasma fluoride levels increase in proportion to the chronic level of fluoride intake. Body fluid fluoride levels are not homeostatically controlled as was once believed (Singer & Armstrong,1960), therefore plasma fluoride levels can be used as an index of previous exposure to the ion (Whitford & Williams,1986).

Systemic fluoride absorption is rapid. A rise in plasma fluoride concentration can be detected within 2-3 minutes of administration. The height of the plasma peak is dependent on the fluoride dose ingested and the rate of absorption, but is also influenced by the total blood volume of the individual. The peak plasma concentration occurs around 30 minutes after ingestion, and is independent of the dose (Ekstrand,1977). The absorptive process occurs by passive diffusion, driven by the concentration gradient. There is, as yet, no convincing evidence that any active transport mechanism is involved. Fluoride is absorbed from both the stomach and the small intestine and much work has been published (Whitford, Pashley & Stringer, 1976; Whitford, Callan & Wang,1982), documenting the influence of pH on the absorptive process. It has been postulated that fluoride is absorbed as the weakly dissociated acid, HF, which has a pKa of 3.45: the uncharged HF molecule would pass through biological membranes much more readily than ionised H^+.

By plotting the plasma concentration of fluoride as a function of time against a semi-logarithmic scale, three exponential phases can be distinguished i.e. an initial increase, followed by a rapid fall for about one hour and thereafter a slower decline. To describe the pharmacokinetics of fluoride, a two-compartment model has been used, where the compartments represent theoretical spaces, conjectured to account for the experimental finding that drugs are distributed into different body fluids and tissues at various rates. In a two-compartment open model, fluoride enters the system via the central compartment and is

then distributed to the peripheral compartment. Elimination of the ion occurs from the central compartment.

The initial increase in the plasma fluoride concentration reflects the absorption of fluoride from the gastrointestinal tract into the circulation. A straight line indicates a first-order process, which means the amount absorbed per unit time is proportional to the amount present (Ekstrand,1977). When the plasma peak is reached the absorption gradually decreases, and the distribution of fluoride from blood into the tissues increases. The falling portion of the curve is usually separated into two exponential phases. The early phase is termed the distribution or α-phase, and the latter the elimination or β-phase. During the α-phase, the fluoride is distributed rapidly to well-perfused tissues, such as the heart, kidneys, liver and to bone, all of which are part of the central compartment. A much slower distribution to the more poorly perfused tissues of the peripheral compartment, such as resting skeletal muscle and adipose tissue, then occurs until a steady state is reached. Thereafter, the curve enters the β-phase, in which it is monotonic, but with a much less pronounced slope. This decline in the plasma fluoride concentration reflects the elimination of fluoride from the body, and is characterised by the elimination half-life ($t = ½$). The plasma half-life for fluoride in human adults, which is variously quoted as 4-10 hours, is calculated from this final exponential slope.

Approximately 50% of a dose ingested by an adult will be excreted via the urine. The amount of fluoride entering the renal tubules per unit time is governed by two factors, namely the glomerular filtration rate and the plasma fluoride concentration. The amount of fluoride excreted in the urine, however, has consistently been found to be less than the amount which enters the kidney tubules (Whitford, Pashley & Stringer,1976). Thus, fluoride handling by the kidney is characterised by filtration at the glomeruli, followed by a variable degree of tubular reabsorption.

In addition, therefore, to the level of chronic fluoride intake in the diet and dentifrices, plasma levels are influenced by the relative rates of accretion and dissolution, and by renal clearance. Most of the remaining 50% will be taken up by the calcified tissues and fluoride will be released from bone during normal remodelling, or during substantial alterations in daily fluoride exposure. In the long term, there is a direct relationship between the concentration of fluoride in plasma and in bone.

1.4 Saliva.

1.4.1 Salivary Glands.

Prior to the end of the 17th century, and the publications of Wharton and Stenson, salivary glands were thought to be excretory organs with a sieve-like role in straining of excrementous substances from the blood, especially the evil spirits of the brain (Garrett,1975). With the realisation that these glands could form external secretions, physicians who practised according to Galen's popular premise that disease was a morbid state of the four "principal humours" (phlegm, blood, yellow bile and black bile) employed salivation as one of their therapeutic strategies. Until the 19th century it was not uncommon for practitioners to prescribe massive doses of calomel (mercurous chloride) to 'cleanse the system' by causing saliva to pour from the mouth.

Saliva is produced by three pairs of major salivary glands and a large number of minor glands situated in the mucosa, particularly of the palate, cheeks and lips. The secretion from each of the glands is both qualitatively and quantitatively distinct, and all are subject to physiological variation. Under resting conditions, different areas of the mouth are bathed in saliva from different glands. For example, the buccal surfaces of the molars and premolars will be covered in parotid saliva; the floor of the mouth, the ventral surface of the tongue and the lingual aspects of the lower anterior teeth will be in contact with mixed submandibular and sublingual saliva, and the palate and the labial mucosa will be coated in

saliva secreted by the minor salivary glands. This regional variation has important implications for the clearance of a variety of substances from saliva, and also for the site-specificity of a number of dental/oral diseases, particularly dental caries.

The submandibular and sublingual glands contain both mucous and serous acini, and can therefore produce a mixed secretion. The minor salivary glands produce a mainly mucous secretion and the large parotid glands consist predominantly of serous acini, producing large volumes of serous saliva on stimulation. The secretions of these various glands enter the mouth at different sites and their distribution and mixing is regulated by functional parameters. It is now evident (Weatherell *et al.*1986), that substances are cleared from saliva at significantly different rates, depending on anatomical site, and it has also been determined that the thickness of the salivary film is of the order 70 - 100 μM(Collins & Dawes,1987). Consequently, all reactions between salivary components on the one hand, and tooth surfaces, bacteria and oral mucosa on the other, occur at solid-liquid or liquid-air interfaces, and are thus subject to surface chemical and physical laws.

The major functions of saliva can be divided into two broad categories: (1) digestive - the various interactions with food, and (2) protective - the interactions with the teeth, soft tissues and the oral microflora. These two sets of functions are associated with a division of labour by the salivary glands. The food-related activities are an overt response to reflex stimulation by olfactory, gustatory and masticatory stimuli. Protection, on the other hand, is afforded by basal secretion, the ongoing, low-level generation of a minimal salivary flow in the absence of overt stimulation (Suddick & Dowd,1980). More specifically, saliva has a primary role in keeping the oral cavity moist, maintaining the health of the oral mucosa, facilitating speech and lubricating food for chewing and swallowing. It is essential for taste acuity and oral hygiene, and it has important protective and antibacterial functions, including the promotion of wound healing via the secretion of epidermal growth factor (Starkey & Orth,1977) and secretory immunoglobulins, such as IgA (Tenuvuo *et al.*1982). These

protective functions are discussed further in Section 1.4.3.

1.4.2 Regulation of secretion and composition.

It has been recognised since 1850 that salivary secretion is under the control of the autonomic nervous system. It is now known that this primarily involves the centrally co-ordinated formation of parasympathetic impulses which evoke most of the fluid secreted, induce contraction of myoepithelial cells, and cause varying degrees of exocytosis. Stimulation of the parasympathetic system generally leads to secretion of water and electrolytes and thus influences the salivary volume, whilst stimulation of the sympathetic system is largely responsible for alterations in the protein concentration (Shannon, Suddick & Dowd,1974).

The average value for the flow rate of unstimulated saliva has been published in a number of studies (Becks & Wainwright,1939; Shannon & Frome,1973; Dawes,1987), and all are in reasonable agreement that mixed, unstimulated saliva is produced at an average rate of 0.31-0.32 ml/min. However, all the aforementioned studies also report a very large standard deviation, indicating a high level of individual variation. A wide range of factors influence the secretion rate of unstimulated saliva, including the level of body hydration, biological rhythms, smoking and numerous drugs such as anti-depressants, anti-histamines, anxiolytics, anti-arrhythmics and diuretics.

In a study carried out on dental students (Dawes,1987) regarding the effects of atropine on salivary flow, subjects were asked to note when the onset of dry mouth symptoms began. On average, this was noted when the salivary flow had fallen by 40-50% of the normal value, in spite of wide variations in flow rate. It was concluded that it was the relative change in the amount of saliva, rather than the absolute values, which was important in producing xerostomia. Stimulated salivary flow rate is influenced by mechanical stimulation, gustatory stimulation, and gland size. It also shows wide individual variation, but the effects of ageing,

once thought to be a flow-reducing factor, have been questioned in recent years (Billings,1993).

The typical quoted inorganic and organic composition of unstimulated saliva is listed in Table 1.1. These figures are subject to enormous variablility, particularly when salivary flow is stimulated. In addition, there are differences between duct saliva and whole, mixed saliva for a variety of constituents, with one of the most significant differences being the concentration of fluoride. This is largely because mixed, unstimulated saliva contains significant quantities of food debris, oral micro-organisms and desquamated epithelial cells, which can contain large amounts of fluoride, all of which are diluted with salivary stimulation. In contrast to other halogens (Stephen,1974), the fluoride concentration of duct saliva is unaltered by stimulation (Oliveby *et al.*1989).

Table 1.1 **Concentrations of unstimulated & stimulated salivary constituents: the values (± S.D. where appropriate) are representative of those in the literature*.**

	Mixed		Submandibular		Parotid	
Constituent	Unstimulated	Stimulated	Unstimulated	Stimulated	Unstimulated	Stimulated
Calcium (mmol/L)	1.56 ± 0.06	1.48 ± 0.04	1.56 ± 0.45	1.92 ± 0.86	0.75 ± 0.25	0.82 ± 0.55
Bicarbonate (mmol/L)			2.2	35.5	1.1 ± 0.1	29.5 ± 9.6
Phosphate (inorganic) (mmol/L)	6.14 ± 0.61		3.6 ± 0.7	5.5 ± 4.0	3.4 ± 1.4	3.3 ± 2.6
Fluoride (µmol/L)	15 ± 0.7	0.56 ± 0.25			1 ± 0.2	1 ± 0.2
Sodium (mmol/L)	6.2 ± 0.5	26.4 ± 11.8	2.6 ± 2.7	45.5 ± 23.4	2.6 ± 2.0	54.9 ± 16.9
Chloride (mmol/L)	17.4 ± 1.4	29.0 ± 8.8	11.9 ± 2.3	23.4 ± 11.3	24.8 ± 7.6	33.3
Protein (g/L)		2.8	1.14 ± 0.58	1.54 ± 1.14	2.35 ± 3.87	1.64 ± 0.51
α-Amylase (g/L)	0.38 ± 0.32		0.25 ± 0.06		1.03 ± 0.11	0.95 ± 0.15
Lysozyme (mg/L)	264 ± 25	62 ± 9		15 ± 9	42 ± 9	23 ± 14
Albumin (mg/L)						2.8
Total solids (% w/v)	6	0.53			0.72 ± 0.24	0.92 ± 0.26
pH		7.08 ± 0.31	6.73 ± 0.45		5.92 ± 0.51	7.67 ± 0.18

(*Waterhouse, Beeley & Mason (1990) Chapter 1, Introduction & Biological Basis. In: *Oral Manifestations of Systemic Disease,* 2nd Ed. Jones J.H & Mason D.K. London, Baillière Tindall. 1-29.)

1.4.3 **Protective functions of Saliva.** (see also Section 3.1)

The protective functions of saliva fall into three main categories: mechanical, anti-bacterial and anti-caries. The mechanical protection awarded by saliva is largely a function of salivary mucins, which are hydrophilic and can entrap large quantities of water. The rheological properties of mucous glycoproteins include low solubility, high viscosity, elasticity and adhesiveness, which enables them to concentrate on the oral mucosal surfaces, where they form an effective barrier against desiccation and environmental insult (Tabak *et al.*1985). These properties enable them to resist dehydration and maintain a well-lubricated and moist mucosal surface. More recently proline-rich glycoproteins have been shown to be extremely effective lubricants, when complexed with salivary albumin (Hatton *et al.*1985). The distribution of this complex in the oral cavity remains to be established, but it could be found on the teeth, as part of the pellicle, and also on the oral mucosa. The anti-bacterial functions are mediated via a wide range of mechanisms, including inhibition of bacterial cell adhesion by secretion of immunoglobulins such as IgA (McNabb & Tomasi,1981) and bacterial lysis by enzymes such as lysozyme, lactoferrin and lactoperoxidase (Tenuvuo *et al.*1981).

1.4.4 **Anti-caries Properties of Saliva.**

The anti-caries properties of saliva are largely mediated via its inorganic constituents, and are demonstrated graphically by the rampant disease encountered in patients with considerable salivary hypofunction. The concentration of ions in saliva varies greatly, as discussed in Section 1.4.2, between the source of saliva, the degree of stimulation, time of collection, and between individuals. Many of the ions complex with other salivary components such as proteins, phosphate and bicarbonate ions. The degree of saturation, however, is determined by the concentrations of uncomplexed ions in solution. Saliva *in vivo*, is invariably supersaturated with respect to fluorapatite and hydroxyapatite, and this supersaturation is a prerequisite for the preservation of dental mineral. Any apatite crystal in

this supersaturated solution will tend to grow, as evidenced by the propensity of partly demineralised crystals to remineralise, in white-spot enamel lesions. However at low pH, saliva becomes undersaturated with respect to hydroxapatite, which therefore dissolves, whilst remaining saturated with respect to fluorapatite. Enamel exposed to such conditions will rapidly develop a carious lesion.

Theoretically, saliva is a poor remineralising solution, as only approximately 0.003% of the volume of saliva consists of mineral. If the saliva were not continually replaced, this mineral would be depleted rapidly at the expense of growing apatite crystals, and supersaturation conditions would exist only briefly. It is possible that the supersaturation of saliva in the enamel pores is too low to survive diffusion through the outermost enamel for crystal growth to occur in subsurface enamel. Thus, remineralisation *in vivo* occurs most readily at the enamel surface, although complete remineralisation of a lesion has been demonstrated *in vitro* (Larsen & Fejerskov,1989).

In the oral environment, proteins may block pores in the enamel and restrict diffusion of mineral ions into the lesion, preventing complete remineralisation. However, clinically it is not important to remineralise a lesion completely : it is sufficient to arrest its further development by depositing a non-reactive mineral such as fluorapatite within the surface layer.

The concentration of fluoride in saliva, and particularly at the enamel surface interface, has a critical role to play in the remineralisation/demineralisation balance. It is largely agreed that the level of plaque fluid fluoride *in vivo,* is dependent on the concentration of fluoride in whole saliva, as opposed to duct saliva, and that plaque bacteria and surface enamel fluoride can also replenish any depletion in plaque fluid concentration. When the pH at the enamel surface is lowered from a physiological value of 7 to around pH 5, the solubility of enamel increases approximately 50 times (Larsen,1975). Although the concentration of free calcium

and phosphate also increases, this is not sufficient to counteract the dissolution of enamel under such conditions. The only factor which does have an influence on the amount of mineral lost from the enamel, is the concentration of available fluoride. When this is adjusted to approximately 1 ppm, the aqueous phase is still saturated with respect to hydroxapatite, but highly supersaturated with respect to fluorapatite. A high supersaturation cannot persist in contact with its solid phase for an indefinite period of time: a precipitation and/or a crystal growth occur, tending to render the solution saturated. When all the fluoride is removed from the aqueous phase by fluorapatite formation, the conditions of supersaturation no longer exist. However, due to precipitation of calcium and phosphate, the aqueous phase is undersaturated with respect to hydroxyapatite and therefore the solid re-dissolves. The specific chemical and physical properties of saliva responsible for its profound anti-caries efficacy are discussed further in Section 3.1.1 and 3.1.2.

1.6 Summary and Aims of Thesis.

In summary, although caries has been recognised as a multifactorial disease for some time, the relative contributions of diet, oral microflora, enamel integrity and fluoride intake on disease progression, have yet to be elucidated. The fluoride concentrations in dental products continue to be empirical, and the frequency of application is often a matter of convenience. Evidence is accumulating (Featherstone *et al.*1990) which indicates that frequent applications of relatively low fluoride concentrations may prove most efficacious which, if confirmed, would enable fluoride therapy to be provided without the deleterious side-effects of high dose regimens. It is, therefore, of paramount importance that the effect of low fluoride levels on enamel re-/demineralisation continue to be investigated, and that those factors which influence salivary fluoride concentration be determined.

In view of the above, the main aims of the work reported in this thesis were:

1). to establish the influence of the fluoride concentration of a range of dentifrices on the

ambient salivary fluoride levels and, in addition, to monitor any concurrent changes in plaque and plasma levels;

2). to determine if ambient fluoride concentration changes *in vivo* could affect the re-/demineralisation of artificial caries lesions *in vitro,*

and

3). to investigate factors which may influence intra-oral fluoride clearance, and which may therefore be responsible for maintaining the anticipated altered intra-oral concentrations.

Chapter 2. Fluoride Measurements

2.1 Introduction

Over the past three decades, a wide range of analytical techniques have been proposed for measuring fluoride concentrations. As technology has developed, and the concentration of fluoride considered therapeutic has diminished (Featherstone *et al.*1990; Damato, Strang & Stephen,1991) so the precision and accuracy of these techniques have improved. Details of the major developments in fluoride analytical techniques are included in Table 2.1. This chapter discusses the most widely accepted methods of fluoride analyses and assesses the advantages and disadvantages of each.

2.2 Early Developments : the Colorimetric Techniques.

Early techniques for fluoride analysis e.g. Willard & Winter (1933), centred around colorimetric methods. These depended on the bleaching of zirconium-alizarin complexes by fluoride ions, resulting in a colour loss. All the colorimetric techniques were prone to interference from complexing ions, and therefore required time-consuming purification by distillation prior to analysis. In addition, a decrease in colour was not a particularly satisfactory parameter for quantification, and such limitations of the early techniques led to their redundancy.

Zirconium/alizarin complexation was superseded by an improved system, using red chelates of lanthanum, which formed a blue complex with fluoride. This colour change occurs when minute quantities of fluoride are present, and results in an intense violet-blue colour, which is highly amenable to colorimetric determination. Distillation remained as the purification technique, but great difficulty was encountered ensuring the distillation apparatus was fluoride-free, and the technique, again, proved very time-consuming.

Table 2.1 . Developments in fluoride analytical techniques.

1924	de Boeur.	Bleaching zirconium/alizarin lakes : negative colour change.
1933	Willard & Winter.	Volumetric method of fluoride determination.
1957	Fremlin, Hardwick & Suthers.	Fluorine^{18} labelling.
1959	Belcher, Leonard & West.	Distillation + alizarin/lanthanum : positive colour change.
1962	Wharton.	Distillation of HF + ZrSPANDS bleaching.
1966	Frant & Ross	Fluoride ion-specific electrode: potentiometry.
1967	Baulmer.	Improved microdiffusion + alizarin complexation.
1968	Fresen *et al.*	Fluoride determination by gas chromatography.
1968	Grøn, McCann & Brudevold.	Determination of fluoride in saliva by F electrode.
1968	McCann.	Comparison of diffusion/colourimetric techniques and F electrode electrode.
1968	Singer & Armstrong.	Fluoride analysis of bone : ashed v unashed samples.
1968	Taves.	HMDS-diffusion + F electrode v fluorescence.
1970	Fry & Taves.	Modified diffusion v direct diffusion + F electrode.
1972	Hall *et al.*	F electrode + known addition.
1974	Venkateswarlu.	Reverse extraction.
1975	Venkateswarlu.	Hanging drop electrode.
1976	Hallsworth *et al.*	Detection of subnanogram amounts with F electrode.
1977	Tsunoda *et al.*	Detection of subnanogram amounts with M.A.S. spectrophotometry
1985	Retief *et al.*	Comparison of F electrode with gas chromatography.
1985	Tyler & Comer.	Fluoride/pH electrode differential cell.
1986	Potter, Hilliker & Breen.	Determination of F and MFP by ion chromatography.
1987	Vogel *et al.*	Microelectrode techniques for fluoride in oral fluids.
1988	Weetman, Geddes & Strang.	Isotachophoresis: simultaneous F, MFP & P determination.

In 1962, Wharton published an article entitled "Isolation and Determination of Microgram Amounts of Fluoride in Materials containing Calcium and Orthophosphate", detailing a method of fluoride analysis which became standard for the ensuing four or five years.

Wharton's method depended on the gaseous diffusion of the undissociated acid HF, and its subsequent trapping in NaOH, thus concentrating the fluoride before final spectrophotometric quantification. This important preparative step, namely the extraction of a substance and its subsequent concentration by diffusion, prior to final analysis, represented a major improvement on contemporary techniques.

In addition to changes in the purification methodology, Wharton also substituted a Zr SPADNS (zirconyl chloride octahydrate, 4,5-dihydroxy-3-(p-sulfophenylazo) -2,7-naphthalene-disulfonic acid, trisodium salt) colorimetric system for the lanthanum system. This had the additional advantages of a single reagent method, in that it was easily prepared, and was considerably more stable. The development of microdiffusion rendered the ashing of most samples e.g. bone, urine and dental materials, unnecessary. This obviated the considerable contamination problem which had plagued this technique during sample incineration in muffle furnaces. The result of all these refinements in technique was a method with an accuracy and reproducibility of ± 0.05 μg fluoride at the 1 μg level, which represented a significant improvement on earlier means. To this day, prior extraction remains an integral part of a number of currently advocated techniques.

When Frant & Ross (1966) produced the ion-specific fluoride electrode, an alternative to the early colorimetric techniques became available. This electrode did not require any tedious distillation to remove contaminants, as the major interfering ions (Al^+, OH^-) could be complexed with suitable buffers. Nor did it require any adjustment of sample volume to produce a sample concentration within a specific range.

In 1968, Taves described a new procedure for achieving the rapid diffusion of radioactive

fluoride ($^{18}F^-$) at room temperature. It involved direct introduction of a simple silicone, hexamethyldisiloxane (HMDS), into the acidified sample employed for diffusion. HMDS is presumed to enhance the rate of diffusion of fluoride by the formation of trimethylfluorosilane (TMFS), which is highly volatile (b.p. 16.4° C) and hydrophobic, thus readily escaping from the aqueous acidic solution. The alkaline absorbing solution decomposes TMFS, trapping fluoride and releasing trimethylsilanol (TMS). Thereafter, two molecules of TMS recondense to form the parent HMDS, which returns to the acidic solution to repeat the cycle of events, resulting in a rapid diffusion of fluoride. Taves (1968) calculated that, under the conditions of this technique, 80 nmoles of HMDS brought about the diffusion of 45,000 nmoles of fluoride from bone in one hour, which was less than one tenth of the time taken by previous diffusion methods.

Diffusion at room temperature also had the advantage of decreasing the likelihood that other volatile components would contaminate the trapping solution. This microdiffusion technique continues to be favoured by a number of workers, as the most convenient and effective method of fluoride extraction prior to analysis.

A variety of other modifications of the fluoride-specific electrode technique have been advocated, e.g. reverse extraction (Venkateswarlu,1974), microanalysis (Vogel, Chow & Brown,1983) and HCl/pH electrode (Tyler & Comer,1985), and are discussed in subsequent sections. Gas chromatography, molecular absorption spectrometry, ion chromatography and isotachophoresis have all been advocated for fluoride analysis, although these techniques enjoy less wide-spread support than the ion-specific electrode methods.

2.3 Potentiometry : The Fluoride Specific Electrode.

2.3.1 Theory.

Four essential components are required to complete an ion-selective measurement :

A sensing electrode (half cell)

A reference electrode (half cell)

A readout device (meter)

A solution containing the relevant ion.

When a sensing electrode is exposed to a sample solution of ions for which it is selective, a potential develops across the sensing membrane surface. This membrane potential varies with the concentration of the ion being measured, and the magnitude of this potential, expressed as a voltage, is also related to the concentration. To make a measurement, a second unvarying potential against which the membrane potential may be compared, is required. This is the function of the reference electrode. An appropriate equitransferrent filling solution, e.g. AgCl, completes the electrical circuit between the sample and the internal cell of the reference electrode. The point of contact between the sample and the filling solution is known as the liquid junction, and is a critical factor in the electrical circuit. A suitable meter serves as the readout device to display the voltage difference of the electrode pair, usually in millivolts or in concentration units. The sample, or during calibration an appropriate standard, is the final component of the circuit.

The fluoride electrode is a solid-state electrode and consists of a section of single-crystal rare earth fluoride, usually lanthanum fluoride, typically 1 cm in diameter and 1 to 2 mm in thickness. The section is sealed to the end of a rigid P.V.C. tube with an epoxy cement. It is critical for the correct functioning of the electrode that this seal is completely watertight.

The crystal is an ionic conductor in which only fluoride ions are mobile. When the membrane is in contact with a fluoride solution, an electrode potential develops across the membrane. The measured potential, corresponding to the level of fluoride ions in the solution, is described by the Nernst equation:

$$E = E_0 - S \log A$$

where

E = measured electrode potential

E_0 = reference potential (a constant)

A = fluoride level in solution

S = electrode slope

The level of fluoride, "A", is the activity or "effective concentration" of free fluoride ions in the solution. The total fluoride concentration, "C_t", may include some bound or complexed ions in addition to the free ions. The electrode however, can only respond to the free ions in solution. The activity of the fluoride ion in a given solution is influenced by a number of different factors including pH, total solution ionic strength, temperature and degree of complexation.

2.3.2 Technique.

It is vital that all standard solutions and all samples are equivalent, in terms of the above parameters, to ensure changes in the measured potential are an accurate reflection of changes in the fluoride ion concentration. The addition of known amounts of a total ionic strength adjusting buffer has the combined effects of removing any potential complexing ions, adjusting the pH to 5.3, and producing a standardised total ionic strength in solution.

Most standards and samples are measured at room temperature (~18°C), and this should be monitored regularly and the sample temperature adjusted as necessary.

A standard calibration curve is plotted on semi-logarithmic graph paper, with the fluoride concentration on the semi-logarithmic axis and the response millivoltage on the linear axis. It is generally accepted that the response of the fluoride electrode to changes in fluoride ion concentration is linear when the fluoride concentration is above 0.1 ppm (Orion,1971). Under these circumstances readings in concentration units can be taken directly from the recording meter. At concentrations below 0.1 ppm F however, the electrode begins to respond in a non-linear fashion and a millivolt reading plotted against a calibration curve is required. A large number of standard solutions should be employed when working at sub-ppm F levels, to ensure an accurate representation of this non-linear section of the curve, and the inclusion of a fluoride-free blank solution is recommended. In addition, the response time of the electrode is significantly increased at low concentration levels. It has been demonstrated (Duckworth, Morgan & Murray,1987) that a designated response time, e.g. 5 minutes, produces a more reproducible result than waiting through an ill-defined period of slow electrode drift.

A further significant problem, applicable to the measurement of any trace substance, is contamination. The preparation of standards by serial dilution must be carried out meticulously. Hence, all samples and standards must be measured in increasing concentration to minimise any potential carry-over, and to reduce the influence of the so-called "electrode memory". Furthermore, the electrodes should be rinsed with deionised water and blotted dry between measurements.

Disposable plastic-ware should be employed wherever possible to eliminate any interaction between the fluoride ions and glass (Hattab,1981). When the use of glass-ware is unavoidable, it must be cleaned in advance with dilute acid, either 0.1M HCl or perchloric

acid, and then rinsed in deionised water. Fluoride standards and samples should never be stored for prolonged periods in glass containers (Hattab,1981). The protocol for fluoride measurements followed during the course of the work reported in this thesis is included as Appendix 3.4.

2.3.3 Summary: Advantages and Disadvantages.

The introduction of the fluoride specific electrode was undoubtedly a breakthrough in the field of fluoride analysis. The tedious process of distillation, required to remove contaminants, was replaced by a simple and convenient buffering system, and the range of sample concentrations which could be detected was extended below 0.1 ppm F (5 μm/L). In addition, the fluoride electrode could be used in conjunction with a conventional millivolt meter, which rendered the cost of the technique minimal. At high concentrations, e.g. > 1 ppm, sample readings can be recorded within 30 seconds and even at concentrations < 0.1 ppm, a 5 minute equilibration time provides reproducible results (Duckworth, Morgan & Murray,1987).

The greatest disadvantage of the fluoride electrode is its deviation from a linear response at extreme low levels i.e. < 0.02 ppm. However, this problem can be overcome if sufficient standards and a blank solution consisting of the appropriate buffer and distilled, deionised water are included in the calibration procedure. It should be emphasised at this point that the ion-specific fluoride electrode responds only to the free ionic fluoride in solution and, if a measure of *total* fluoride, i.e. covalently bound or complexed fluoride plus ionic fluoride is required, either some appropriate pre-treatment of the sample is necessary, or an alternative technique should be employed.

2.4 Potentiometry: The pH Electrode/Fluoride Electrode Differential Cell.

2.4.1 Theory.

In 1985, Tyler & Comer published details of a fluoride analytical technique which they claimed extended the range of the fluoride specific electrode by one decade, towards the lower limit of detection. The apparatus required was a microprocessor digital analyser, with two high impedance outputs, in this instance the Orion 901 Ionalyzer, a fluoride specific electrode (Orion 94-09) and a flat surfaced combination pH electrode (Orion 91-35).

Tyler & Comer based their technique on the premise that the electrochemical analysis of fluoride may be conducted at pH values below the pK of 3.2 for hydrofluoric acid. Under these conditions, HF will be the predominant fluoride-containing species, and the potential difference between a combination pH electrode and a fluoride electrode forming a differential cell, is a logarithmic function of the total fluoride concentration.

Standard solutions were prepared from Aristar grade hydrochloric acid (0.1 M) and analytical grade sodium fluoride. The response of the fluoride electrode in combination with the glass pH electrode, in aqueous solutions of varying pH and fluoride concentrations, demonstrated the Nernstian response of the system. Tyler & Comer noted that the results became increasingly independent of the hydrogen ion concentration below pH 2. The comparison of this system with the conventional fluoride/reference electrode system plus TISAB, did indeed show an extension of the concentration range by approximately one decade, to levels of 0.01 - 0.005 ppm F. A greatly reduced response time was also recorded, namely 95 % response within 30 seconds in an 0.005 ppm F sample, and total equilibration within 2 minutes. Data from a reproduction of this technique is presented in Figure 2.1, and would appear to endorse claims about this system.

It is generally accepted that the lower limit of Nernstian response is determined by the finite

solubility of the sensing membrane used in an ion-selective electrode and, within this limit, the fluoride electrode responds to the fluoride released by its' own dissolution. It has been suggested (Lingane,1968) that lanthanum fluoride shows an enhanced solubility in an acid environment, and if this is correct then the increased solubility of the lanthanum fluoride membrane would indicate a higher, not a lower limit for Nernstian response at low pH values. However the reaction

$$H^+ + F^- = HF$$

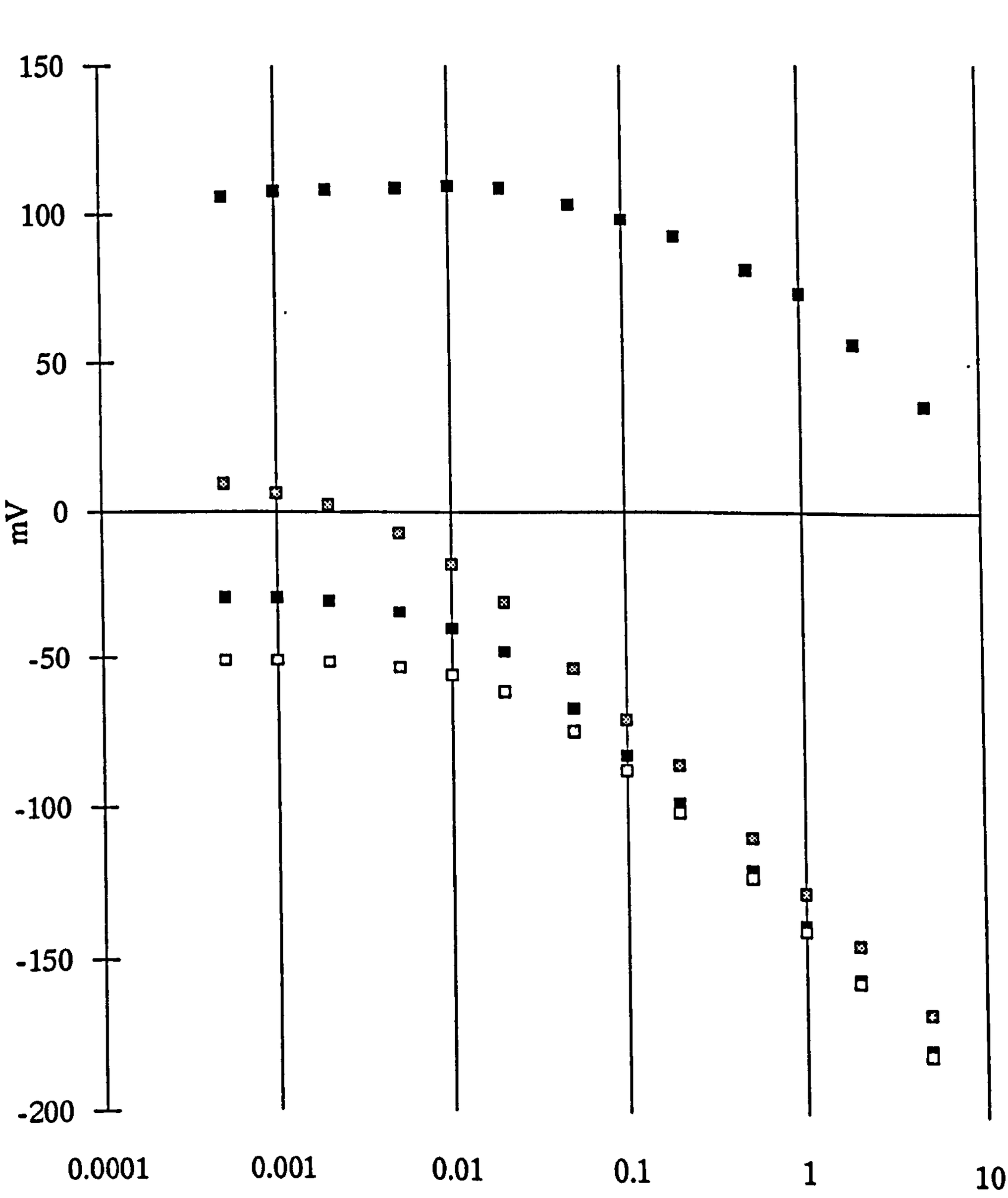
leads to a reduction in the fluoride ion concentration at pH <4 and, accordingly, a solution of 10^{-6} M F^- at pH 5.5 would show a fluoride ion activity of only 10^{-9} M at pH 1.

Tyler & Comer (1985) thus speculated that the lower limit of Nernstian response is determined not only by the solubility of the membrane, but also by the influence of interfering ions. The significant reduction in hydroxide ion concentration, from $10^{-8.5}$M at pH 5.5, to 10^{-13}M at pH 1, may be responsible in part, for the enhanced function of the fluoride electrode at low pHs.

2.4.2 Summary : Advantages and Disadvantages.

The rapid response time of this system, particularly at low fluoride concentrations, should be considered a major advantage where large numbers of samples require analysis. In addition, the extension of the lower limit of the concentration range by a factor of ten may obviate any sample concentration procedure prior to analysis. A significant drawback, however, is the volatility of HF, and it may be that the entire system requires to be enclosed for optimum function.

Figure 2.1 Comparison of the response of the fluoride electrode and the fluoride/pH electrode differential cell to changes in fluoride ion concentration.



2.5 Potentiometry: Microelectrode Techniques.

Investigations into the fluoride concentration in surface enamel and in plaque fluid required the development of analytical techniques which were capable of quantifying fluoride in sample volumes in the range 5-200 μL. In 1983, Vogel *et al.* published details of two techniques, one describing the construction of a microelectrode, and the other an adaptation of the standard fluoride electrode.

The fluoride microelectrode was constructed using the tip of an 'Eppendorf'- type polypropylene micropipette, into which was inserted a sphere of lanthanum fluoride of greater diameter than the pipette tip. The exposed surface of this disc was ground flat and polished to a mirror finish. The electrode was then filled with fluoride electrode

internal filling solution (0.1 M KCl, 10^{-3} M NaF saturated with AgCl) and connected to the electrode meter via a silver-chloride coated silver wire and a shielded cable.

The adaptation of the standard electrode consisted of a nylon cylinder into which seven sample holes were drilled. This was then fitted over the sensing surface of the electrode which was, in turn mounted in the inverted position. Samples could then be deposited into the adapter wells with a pipette, and a glass micro-capillary tube connected to the reference electrode via an agar bridge, is brought into contact with the samples to obtain readings. This device proved to be considerably faster than the miniature electrode since all seven samples under test reached equilibrium with the electrode surface at about the same time.

Subsequent refinements of the microelectrode techniques, such as the immersion of the sample under water-saturated mineral oil, the use of a stereo-microscope and of mechanical micro-manipulators to position equipment and manipulate samples, have improved further their accuracy and facilitated the determination of fluoride in plaque fluid and enamel biopsy samples of 1-2 μl volume. Good correlations between these modified techniques and both

standard fluoride electrode measurements and gas chromatography (Retief *et al.*1985) have been published.

2.6 Gas/Liquid Chromatography.

The details of gas/liquid chromatography were first published in 1941, by Martin & Synge, who described a technique based on the principal that compounds in a mixture migrate at differing speeds when carried along by an inert gas, through a tube which has been packed or treated in a special way. The first application of this methodology for fluoride determination in biological samples was described by Fresen, Cox & Witter in 1968, who advocated this as being significantly more rapid than previously described diffusion techniques. The mechanism is based on the conversion of aryl- or alkylchlorosilane by water, to the corresponding silanol, which then reacts selectively with fluoride to form fluorosilane. This is then extracted from the acidified solution with an organic solvent e.g. benzene, and the trimethylfluorosilane is determined by gas chromatography. Concentrations as low as 0.01 g F^-/mL can be determined rapidly, with a variation of < 5%.

In 1973, Munksgaard & Bruun published details of a method which used gas chromatography to determine the fluoride content of superficial enamel biopsies. They claimed that amounts of less than 1 ng F in a sample volume of 4-6 μL could be detected with a standard deviation ± 2 %. After each determination, around 10 to 15 minutes was required to stabilise the gas chromatographer.

In summary, this is a useful technique when the appropriate equipment is available, although it also requires the inclusion of internal standards and blank solutions for low level measurements. The major disadvantage, however, is the volatility of the TMFS, and great care is necessary to ensure this is not lost during the analytical process.

2.7 Molecular Absorption Spectrometry.

Molecular absorption spectrometry using aluminium fluoride has been advocated as an analytical technique for fluoride for a number of years. Tsunoda *et al.* (1977), described a method whereby the molecular absorption of aluminium fluoride at 227.45 nm, produced in a graphite furnace by mixing 50 μL of pure sample and 20 μL of $Al(NO_3)_3$ aqueous solution (0.01M), was used as the quantifying parameter. A deuterium lamp, or a platinum hollow cathode lamp, was used as the light source for the molecular absorption, and the background absorption measurements, respectively. It has been demonstrated that molecular absorption spectrometry (MAS) can determine organic i.e. covalently-bound to protein, fluoride in addition to ionic fluoride, and it was therefore considered that this method may be suitable for the determination of the total fluoride content of plasma, urine or saliva, without any pretreatment of samples. Comparisons with the fluoride specific electrode seem to validate this hypothesis.(Chiba *et al.*1980).

The analytical range of the MAS technique was 10^{-5}-10^{-6}M (0.19-0.019 μgF/mL), and the sample volume required was only 5 μL. The high protein content (8%) of the plasma samples results in a high viscosity, and increases the sampling error. The necessary compromise between excessive sample dilution with a resultant decrease in fluoride concentration, and the increased sampling error produced by the high viscosity is one of the major drawbacks of this technique.

2.8 Ion Chromatography.

The terms "Ion Chromatography" and "High Performance Liquid Chromatography (H.P.L.C.)" are virtually inter-changeable. Both techniques employ an injector, pump, separation column and a detector. The only difference is the separation mechanism in the analytical column. Classical high performance liquid chromatography separates compounds that are either polar or non-polar, while ion chromatography separates compounds that carry

positive or negative ionic charges. The accepted definition of ion chromatography is a liquid chromatograph technique which utilises ion exchange resins as the means of separation, and conductivity as the means of detection for the analysis of inorganic anions and cations. Whilst techniques for measuring organic ions, such as organic acids, sugars and alcohols, and organic anions such as amino acids, have been available for a number of years, the application of this technology to quantify inorganic ions such as fluoride, is relatively recent.

One of the major advantages of ion chromatography is its ability to quantify both ionic fluoride and monofluorophosphate. In this capacity it has been used widely to determine the bioavailabilty of these species in fluoridated dentifrices. A linear response to the concentration of $NaPO_3F$ is claimed in the range 175 to 552 μg/mL (Chen *et al.*1985) with a correlation co-efficient of 0.999. Where multiple anions are to be analysed, ion chromatography undoubtedly offers a convenient technique.

2.9 Isotachophoresis.

Isotachophoresis is an electrophoretic technique which separates ions according to their mobility in an electric field, which is largely dependent on their molecular weight and ionic charge. The sample ions are introduced between a faster moving 'leading' electrolyte and a slower moving 'terminating' electrolyte, and a constant current is applied to the system. Separation of the ions into zones takes place: these zones remain adjacent to each other in order of their net mobilities (Gower & Woledge,1977). As with HPLC, the major advantage of isotachophoresis is that a number of different ions can be measured in each sample (Weetman, Geddes & Strang,1988). In the dental field, this technique has probably been most widely used to measure the organic acid anion content of plaque (Creanor *et al.*1986; Geddes & McNee,1982), but it has also been advocated for the simultaneous determination of ionised fluoride and monofluorophosphate in dentifrices and saliva (Weetman, Geddes & Strang,1987; Weetman & Geddes,1993). A reproducibility of 0.9997 (n=10) over the

concentration range 0.5-50 mM/L has been reported. However, the technique has a limited number of proponents, mostly due to the relatively high limit of detection (0.2 mmol/L or 0.38 ppm for F^- and 0.3 mmol/L or 0.57 ppm for $NaPO_3F$) and the relative scarcity of suitable equipment.

2.10 Summary.

A broad range of techniques for the analysis of fluoride is currently available. When selecting a particular methodology, the investigator must weigh up a number of relevant factors, specifically the nature of the fluoride species under investigation, the type and volume of sample to be collected, the equipment available, the expense of purchasing alternatives and, finally, the availability of technical assistance. The advent of the fluoride specific electrode has simplified the analytical process greatly, and its relatively low cost and flexibility have made it the method of choice in a wide variety of circumstances. For these reasons, it was the apparatus employed for the fluoride analyses undertaken in the work described in this thesis.

Chapter 3. Studies on Salivary Fluoride Concentration: Review and Preliminary Investigations.

3.1 Introduction: The Anticaries Properties of Saliva.

The complexity of saliva as a biological fluid has led to it being described as the antithesis of a pure chemical compound (Nikiforuk,1985), and the impact of saliva on oral and dental health is demonstrated graphically by the rampant caries, fulminating fungal infections and sensory disturbances experienced in patients with radiation-induced xerostomia (see Sections 1.4.3 and 1.4.4). Furthermore, over fifteen different factors have been identified which can influence the composition of whole saliva (Dawes,1993).Two major groups of salivary characteristics are pivotal in determining the caries susceptibility of an individual: the chemical characteristics and the physical characteristics.

3.1.1 The Chemical Characteristics of Saliva.

The buffering capacity of saliva has been defined by Jenkins (1978) as "the power to resist changes in pH when acid or alkali is added". The buffering capacity of saliva is flow rate dependent (Dawes,1974), being increased at higher flow rates, and correlates well with caries incidence (Muracciole,1955; Agus & Schamschula,1983; Pienikakkinen *et al.*1985). Ericsson (1959) stated "buffering capacity has the best established connection with caries", and Larmas (1993) suggested that buffering capacity is one of the best indicators of caries susceptibility because it is indicative of host response. Ericsson also demonstrated that buffering capacity varied throughout the day, showing a progressive increase until early evening, with a notable increase after meals. This work was substantiated by that of Krasse (1961).

However, the ability of saliva to neutralise pH is only of limited importance if it is unable to

influence the pH at the plaque/enamel interface. Englander *et al.* (1959) measured pH changes and lactic acid concentrations in plaque on the buccal surfaces of molar teeth over a 20 minute period following exposure to a sucrose solution, with initial readings taken with saliva flowing over the plaque. The experiment was repeated, and saliva excluded by cannulating the parotid duct orifices and ensuring good aspiration of saliva from the floor of mouth. Results showed a steeper and more prolonged pH drop and elevated lactate levels when saliva was excluded.

There are two main buffering systems in saliva, of which the bicarbonate system is the most significant. This system is summarised by the following equation:

$$H^+ + HCO_3^- \Leftrightarrow H_2CO_3 \Leftrightarrow CO_2 + H_2O$$

The conversion of HCO_3^- to H_2CO_3 has a pK value of approximately 6.1 (Nikiforuk,1985) and this system provides maximal buffering at the average ionic strength of saliva (Ericsson,1959).

Carbon dioxide is lost from saliva as it exits from the salivary duct. This results from the concentration gradient established between the high levels of CO_2 in ductal saliva and a lower concentration of CO_2 in the oral cavity saliva, where it has been exposed to the atmosphere. Ductal saliva has a pCO_2 of approximately 46 mm Hg and saliva in the mouth, where it has been exposed to air, has a pCO_2 of 0.3 mm Hg (Nikiforuk,1985). Such a loss of CO_2 results in a reduction in the hydrogen ion concentration. This phenomenon has been termed "phase buffering". The presence of carbonic anhydrase in saliva catalyses the reaction:

$$H_2CO_3 \Rightarrow CO_2 + H_2O$$

and will reduce further the hydrogen ion concentration. Dawes (1969; 1974) showed that bicarbonate concentration increases in parotid and submandibular saliva with an increase in

flow rate. Furthermore, levels of bicarbonate are elevated as long as salivary stimulation persists (Dawes & Macpherson,1992).

Lilienthal (1955) found, in a series of experiments where bicarbonate was removed leaving phosphate and proteins as buffering systems, that phosphate had a reduced ability to buffer saliva in comparison with bicarbonate. Removal of the phosphate by dialysis of saliva left only the proteins, which had no discernible buffering effect on its own. The mechanism of the phosphate buffering system has been proposed according to the following equation:

$$H_2PO_4^- + H^+ \Leftrightarrow H_3PO_4$$

Other methods whereby saliva can counteract a fall in pH include the production of ammonia and decarboxylation reactions. Curtis & Kemp (1984) and Cole & Eastoe (1988) discussed the production of ammonia by bacterial metabolism of salivary urea. Ammonia produced at low pH values will counteract the effect of acid, raising the plaque pH. In addition, the decarboxylation of amino acids, with the release of amines , will also raise plaque pH by mopping up protons. The effect of simple dilution on plaque pH is difficult to study, as saliva has such a complex buffering capacity. However, as pH follows a logarithmic scale, it is anticipated that large dilutions would be required before any effect was evident.

Saliva is generally supersaturated with respect to enamel mineral (Grön,1973) and this plays a key role in the inhibition of enamel demineralisation and in the promotion of remineralisation. The degree of saliva saturation with respect to enamel mineral is dependent on the activity of calcium and inorganic phosphate which are, in part, a function of the ionic strength and pH of the saliva (Vogel, Naujoks & Brudevold,1965). These parameters vary between individuals, and within individuals, according to salivary flow rates (Vogel, Naujoks & Brudevold,1965). Lagerlöf (1983) assessed the effects of flow rate and pH on calcium phosphate saturation in human parotid saliva. He found an increase in saturation with respect to hydroxyapatite, tricalcium phosphate and octacalcium phosphate with increasing

flow rates up to 2.0 mL/min. Parotid saliva remained undersaturated or just saturated with respect to dicalcium phosphate dihydrate at all flow rates assessed. At a constant flow rate, the pH was adjusted and saturation with respect to calcium phosphate compounds re-assessed. Saliva became undersaturated with respect to dicalcium phosphate at pH 8.0, octacalcium phosphate at pH 6.75, tricalcium phosphate at pH 6.25 and hydroxyapatite at pH 5.5. In contrast, ionic calcium levels increased as a result of calcium phosphate dissociation.

The presence of a supersaturated solution would be expected to result in precipitation of calcium phosphate salts within the mouth. This does not occur, largely due to the presence of salivary proteins which inhibit precipitation. Hay & Moreno (1989) discussed the curious nature of salivary supersaturation in terms of its ability to remineralise a carious lesion but to cease mineral deposition at a certain point. Furthermore, mineral precipitation does not normally occur in salivary gland acini or ducts, and teeth do not increase in size through mineral acquisition. Speculation began, therefore, about the existence of a specific mineral precipitation inhibitor.

Analysis of the protein fractions of saliva have revealed two types of protein which have been attributed with the ability to inhibit mineral deposition. These have been identified as statherin and a family of eight proteins with a high degree of charge symmetry called the proline-rich proteins (Hay,1990). Statherin is a small tyrosine-rich protein with a highly charged amino terminal and its active component located in the first six residues (Hay & Moreno,1989). The actions of these proteins include the ability to inhibit mineral precipitation *de novo* within the salivary gland system, so-called primary precipitation, and mineral precipitation inhibition by binding to calcium phosphate crystal surfaces, so-called secondary precipitation (Hay,1990). The ability to bind to the surface of enamel but probably not penetrate into the lesion (Hay,1990) would suggest their role as co-ordinators of mineral deposition within the remineralising carious lesion.

The influence of fluoride ions on the solubility of various mineral phases in saliva and plaque fluid is also of profound significance in determining the dynamics of mineral deposition and dissolution. This role of fluoride will be discussed in Section 3.1.3.

3.1.2 The Physical Characteristics of Saliva

Whilst the chemical influences of saliva on enamel mineralisation have been of interest to cariologists for decades, the physical properties have only been extensively investigated over the last 10 years (Weatherell *et al.*1986; Collins & Dawes,1987; Dawes & Macpherson,1993).

Salivary flow rates, both stimulated and unstimulated, were the earliest parameters to be measured, but more recently total salivary volume, salivary film thickness, differential flow rates within the mouth, and salivary film velocity have all been identified and scrutinised as potentially significant factors in determining enamel mineral flux. These investigations, and the interpretation of the results, are complicated by the fact that many of the physical characteristics of saliva, particularly flow rates, have a direct effect on the chemical constituents (Dawes,1969; Dawes,1974; Dawes,1975). Saliva is also subject to the influence of circadian rhythms. Periods of high flow occur in the afternoon and early evening, with a negligible flow through the night. The total volume of saliva secreted throughout a 24 hour period is in the range 700 - 800 mL with a variable contribution from the different salivary glands during stimulated and unstimulated flow(Nikiforuk,1985)The relationship between salivary flow rate and caries experience is difficult to determine. Mandel, (1974) reporting on the study of Shannon & Terry (1965), suggested a difference of 0.005 mL/min in resting parotid flow between high and low DMF groups. The clinical significance of this is highly debatable: however a reduction in an individual's salivary flow is often related to an increase in caries experience (Thakker & Sloan,1990).

The volume of saliva in the oral cavity is not constant. It varies before and after swallowing

and is also affected by the rate of swallowing. Dawes (1983) developed a mathematical model to determine salivary clearance of sucrose from the oral cavity. This model assumed the mouth to behave like an incomplete siphon, such that each time an individual swallows there will be a finite quantity of saliva retained in the mouth. Attempts have been made by Lagerlöf & Dawes (1984) to quantify the volume of saliva in the mouth before and after swallowing, by determining the change in concentration of potassium or chloride ions in samples of distilled water swished around the mouth. The volume of saliva in the mouth before swallowing was found to be approximately 1.0 mL, whilst the volume after a swallow was approximately 0.7 mL. An increase in flow rate gave rise to an increase in the rate of salivary swallowing, and to an increase in the volume of each swallow.

The distribution of saliva in the oral cavity has implications for both the initiation and progression of enamel caries. Salivary distribution has been addressed by Collins & Dawes (1987), who established that saliva forms a very thin film, covering the surfaces of the oral cavity. Measurements of the surface area of the mouth, in conjunction with the average volumes of saliva before and after a swallow, suggest a film thickness of between 0.07 and 0.10 mm. Furthermore, Dawes *et al.* (1989) have shown that such a film moves at different speeds in different parts of the mouth. Rapid movement may be observed in the lower anterior lingual region, whereas slow film movement is observed in the upper anterior labial region. The direction of salivary flow would appear to be towards the posterior regions of the mouth, and ultimately towards the pharynx for swallowing (Weatherell *et al.*1984; Lecompte & Dawes,1987).

This distribution and movement of saliva, whilst extremely important for the chemical processes described previously, also has implications for the clearance of bacterial substrate, plaque accumulation, local mineral saturation and fluoride levels. A variation of distribution and retention of salivary fluoride in different areas of the mouth has been demonstrated using sodium fluoride tablets and sodium fluoride mouth-rinses (Weatherell *et al.*1984; Primosch,

Weatherell & Strong,1986). The differential patterns of fluoride distribution remained at the end of the two hour experimental period (Weatherell *et al.*1984) and it has not yet been established what occurs after this time. Similar patterns are seen for glucose and sucrose (Dawes & Weatherell,1990; Dawes & Macpherson,1993).

The upper labial sulcus seems to be a region of prolonged fluoride retention, which correlates with the rate of flow of the salivary film in this area (Dawes *et al.*1989). Dawes & Macpherson (1993) developed this work by assessing the distribution of sucrose and saliva around the mouth whilst chewing gum and found a very uneven pattern of sucrose distribution. Low sucrose levels were generally found in regions of high film velocity and *vice versa.* The mathematical model of Dawes (1983) identified the unstimulated salivary flow rate and the volume of saliva immediately before and after swallowing, to be the most important parameters in determining the clearance of non-adherent food substances from the oral cavity. It is not known if adherent foods will clear from the oral cavity in the same manner.

The modifying effect of saliva on the local environment of the tooth makes it very difficult to determine the relative effects of remineralising factors. For example, does the ability of saliva to clear bacterial substrate rapidly from the locale of an incipient carious lesion, have as profound an effect on enamel remineralisation as use of a fluoridated dentifrice ? Hence one of the topics investigated in this thesis relates to the role of intra-oral fluoride concentrations on enamel demineralisation and remineralisation.

3.1.3 The Role of Salivary Fluoride.

Over the last 20 years, there has been an evolution in the proposed mechanisms of fluoride activity, and therefore a concomitant shift in the perceived importance of salivary fluoride. At the time when systemic uptake of fluoride from tablets, drops or fluoridated water (and its subsequent incorporation into developing enamel) was considered the primary protective

mechanism, salivary fluoride levels were deemed insignificant. Latterly, however, the topical effects of fluoride have become regarded as paramount, and the role of varying salivary fluoride concentrations, and the duration of salivary fluoride retention have received widespread attention (Oliveby *et al.*1989; Sjögren & Birkhed,1993).

A variety of intra-oral fluoride sources have been proposed as providing the reservoir for the low concentrations of fluoride or 'ambient fluoride' which persists in saliva following topical application (Lagerlöf, Oliveby & Ekstrand,1987; Arends & Christoffersen,1990). These include protein-bound fluoride, plaque fluoride, surface enamel, and CaF_2 which was demonstrated to be deposited on enamel after exposure to high concentrations of fluoride more than 40 years ago (Gerould,1945). More recent studies have shown that this is also the case when dentine or cementum is exposed to fluoride (Saxegaard, Valderhaug & Rölla,1987).

It has also been demonstrated that the topical application of fluoride in *high* concentrations will produce supersaturation with respect to hydroxyapatite (HAP) and fluorohydroxyapatite (FHAP), in the solution phase at the saliva/plaque : mineral interface. These conditions of supersaturation have been shown to enhance enamel remineralisation and to inhibit its demineralisation (ten Cate & Duijsters,1983; Featherstone *et al.*1990), and it is postulated that these effects may be largely responsible for the cariostatic impact of a wide variety of topical fluoride vehicles.

Topical application of fluoride produces a rapid rise in the fluoride concentration of whole saliva, which has been termed the "application phase", followed by a "distribution phase" lasting up to 30 min (Ekstrand, Lagerlöf & Oliveby,1986; Ekstrand,1987; Duckworth, Morgan & Murray,1987), and a more prolonged decline in F concentration, the "elimination phase", which may last for a period of hours (Bruun *et al.*1982). A number of workers have demonstrated a relationship between the fluoride concentration of a topical fluoride vehicle

and the concentration of fluoride found in mixed saliva and plaque up to 2 hours after the fluoride application (Bruun *et al.*1982; Duckworth, Morgan & Murray,1987; Ekstrand,1987). However, *in vitro* studies have shown that fluoride concentrations as low as 0.04 mg/L have a cariostatic effect (Margolis, Moreno & Murphy,1986) and, as a consequence, interest is increasing regarding the influence of the much lower, ambient (i.e. several hours after application) salivary and plaque fluoride concentrations. As detailed above, current regimens of topical fluoride application produce intermittent concentration peaks in saliva of relatively short duration, followed by a more prolonged clearance, with a return to baseline some hours later. Any detrimental side-effects of fluoride therapy, such as gastric erosion and enamel fluorosis, are a result of systemic ingestion/absorption (Angmar-Månsson & Whitford,1984), and it would therefore be advantageous if lower concentrations of topical fluoride could demonstrate a therapeutic benefit. Featherstone *et al.* (1990), have shown an inverse logarithmic relationship between the fluoride concentration of a treatment solution (F conc. ≥ 1 mg/L) and the relative mineral change in a pH-cycling model. The implication of their results is that a large increase in concentration in an already high fluoride solution would not produce a directly proportional effect on mineral, but that a small change in low fluoride concentrations would have a profound effect. In addition, concentrations of fluoride previously considered insignificant, are now known to inhibit demineralisation and enhance remineralisation (Arends *et al.*1983). Therefore the small differences in ambient saliva and plaque fluoride concentrations produced by different dentifrice fluoride concentrations, may be influential. If fluoride levels in the 0.1-0.01 ppm range can be demonstrated conclusively to be caries-inhibitory, then new methods of sustained delivery could replace present, intermittent techniques, with their attendant deleterious side-effects.

Recommendations for the currently accepted methods of topical fluoride utilisation are largely based on the assumption that the therapeutic efficacy of topical fluoride is the direct result of a decrease in enamel solubility, which occurs as a consequence of fluoride uptake

by surface enamel (Zero *et al.*1988). However, attempts to correlate bound enamel fluoride concentrations with reduced caries experience have not been successful (Schamschula *et al.*1979; Retief, Harris & Bradley,1987). It has been proposed subsequently that the cariostatic effects of fluoride are related to the presence of low concentrations of ionic fluoride in the oral environment (Arends & Gelhard,1983; Arends *et al.*1984) and although there is an abundance of evidence to suggest that low concentrations of fluoride decrease the rate of enamel demineralisation (Moreno & Zahradnik,1974; ten Cate & Duijsters,1983) and enhance the rate of enamel remineralisation (Koulourides *et al.*1974; ten Cate & Arends,1977; Silverstone *et al.*1981; Gelhard & Arends,1984; Featherstone *et al.*1986) the precise mechanism of action is, as yet, unknown.

3.2 Aims.

The main aims of this preliminary study were, therefore:

1. to establish the ambient salivary fluoride concentrations of a group of volunteers

and

2. to monitor the changes in ambient salivary fluoride levels which occurred when NaF dentifrices, fluoridated to a variety of concentrations, were introduced.

3.3 Materials & Methods.

3.3.1 Study Population.

Twelve adult volunteers were recruited from clinical and technical staff in the Glasgow University Dental School. The mean age was 32 years and there were 6 males and 6 females. All were dentate, and routinely used a fluoridated dentifrice twice daily. Additional information, including unstimulated salivary flow rate, baseline salivary fluoride concentration and normal toothbrushing habits was collected and is presented in Table 3.1.

3.3.2 Volunteer Protocol.

All 12 volunteers were provided with a sodium fluoride dentifrice containing a silica abrasive supplied by Unilever Research, Port Sunlight, Bebbington, The Wirral, U.K. The fluoride concentrations in the dentifrices were 500 ppm, 1000 ppm, 1500 ppm and a placebo dentifrice which contained 0.22 ppm F. All dentifrices were identically packaged and flavoured. Details of the available fluoride over time are provided in Appendix 3.1. A detailed protocol (Appendix 3.2) for use of the dentifrice was issued to all volunteers and they were instructed to refrain from using any other fluoride-containing product for the duration of the study. All subjects were issued with each of the dentifrices in random order.

3.3.3 Experimental Protocol.

Baseline salivary samples were collected from each volunteer and a note made of their stated routine toothbrushing habits. After familiarising themselves with the volunteer protocol, the subjects were allocated dentifrices in a random order. Mixed, unstimulated saliva aliquots were collected on alternate weekdays by the following procedures: volunteers were seated comfortably and asked to swallow prior to beginning sample collection. They were then asked to spit into pre-weighed bijou bottles, every half minute for five minutes, all the saliva which had collected behind their closed lips. Salivary flow rates were determined by weight and not by volume, as measurement by volume has been shown to be less reliable (Navazesh & Christensen,1982). Samples were collected at midday, to maximise the time since last toothbrushing and last food intake, and were stored at -16°C until required for analysis. Further details of the saliva collection technique are provided in Appendix 3.3. Each dentifrice was used for a period of 4 weeks and the experiment ran for a total of 16 weeks.

3.3.4 Analytical Techniques.

The frozen saliva samples were allowed to return to room temperature (~18°C) over a 2

hour period. They were subsequently vortex-mixed to redistribute any particulate material.

Equal volumes of saliva were mixed with Total Ionic Strength Adjusting Buffer (TISAB, *Orion Research Incorporated Ltd.*, Massachusetts, U.S.A.) and pipetted into microwells obtained from the same company. A fluoride-specific electrode (*Orion Research 94-09*) and a suitable reference electrode *(Orion Research 90-01*) were then introduced into the sample, which was agitated continuously by magnetic stirring. Millivolt readings were taken after a 10 minute period, as it was found, in agreement with Duckworth *et al.,* (1987), that a finite equilibration time yielded more reproducible results than an indefinite period of electrode drift (see Section 2.3.1). Suitable standard solutions were prepared in accordance with Appendix 3.4, and incorporated into the measuring procedure. The electrodes were recalibrated every 3 hours. The millivolt readings were then plotted against fluoride standard concentrations on semi-logarithmic graph paper and fluoride concentrations of the sample determined.

3.4 Results.

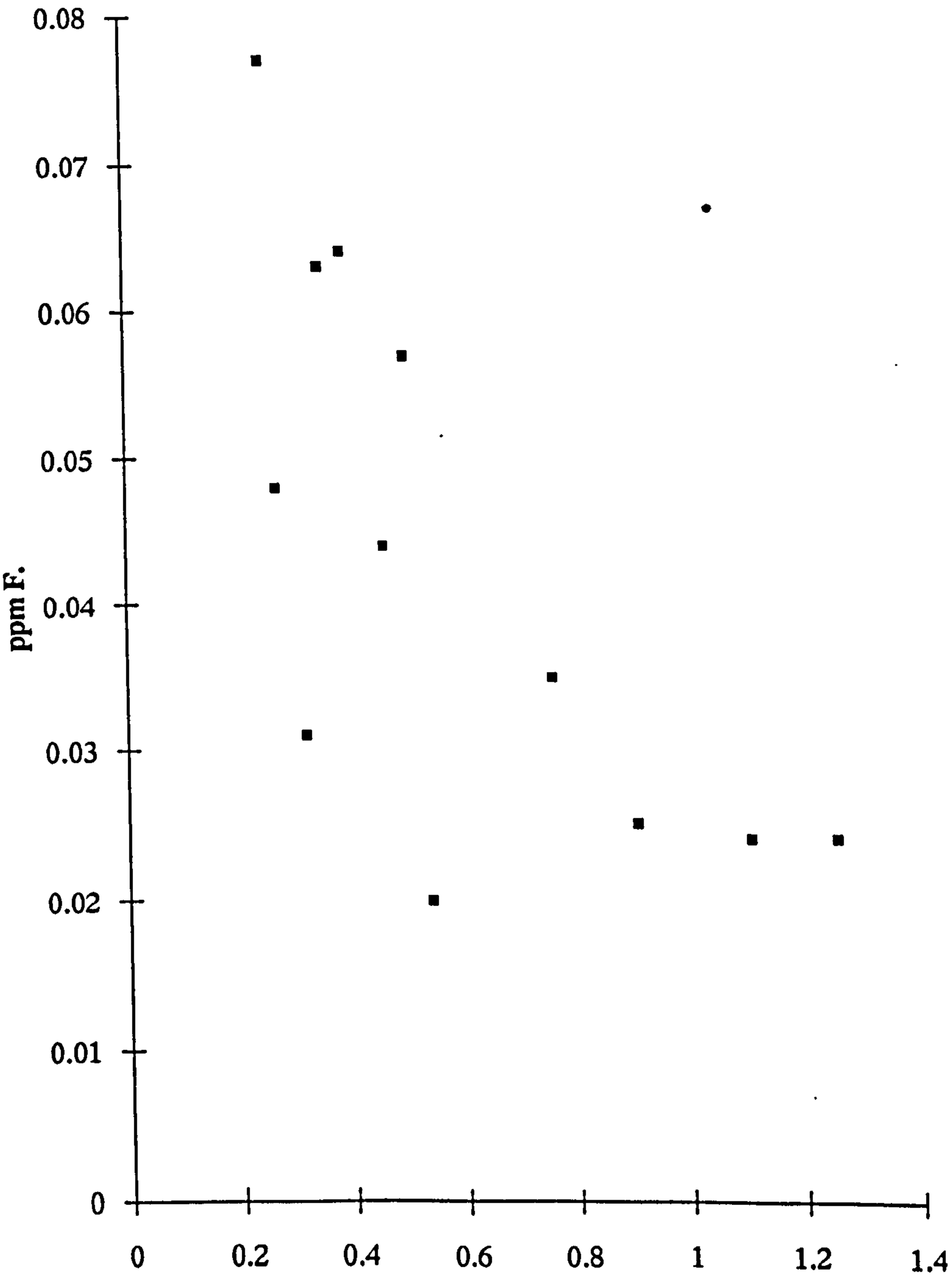
3.4.1 Demographic Data.

The preliminary data collected on the study population are compiled in Table 3.1, and demonstrate that the unstimulated salivary flow rate varied between individuals from 0.27 g/min to 1.26 g/min. Whilst these values lie within the normal range, it was anticipated that those subjects at the extremes of this range might have significantly different ambient salivary fluoride levels as a result of their particular patterns of intra-oral clearance. In fact, Figure 3.1 shows the unstimulated salivary flow rate (g/min) plotted against salivary fluoride concentration, and would appear to suggest a trend towards higher fluoride levels with lower unstimulated salivary flow rates. Analysis of these data revealed a moderate negative correlation of 0.68, suggesting that unstimulated salivary flow rate does influence ambient salivary fluoride concentrations.

Table 3.1 **Unstimulated salivary flow-rate, baseline salivary fluoride levels and toothbrushing habits for all 12 subjects**

Volunteer	Salivary flow rate (g/min)	Baseline salivary fluoride concentration (ppm F)	Tea consumption (units/daily)*	Post-brush rinsing
1	0.32	0.031	3-4 units	Yes
2	1.11	0.024	nil	Yes
3	0.54	0.020	nil	No
4	1.26	0.024	4 units	Yes
5	0.25	0.077	3 units	Yes
6	0.50	0.057	2 units	No
7	0.39	0.064	3 units	Yes
8	0.46	0.044	7 units	Yes
9	0.35	0.063	2 units	Yes
10	0.91	0.025	nil	Yes
11	0.76	0.035	1 unit	No
12	0.27	0.048	nil	No

Figure 3.1 Unstimulated salivary flow rate (g/min) versus baseline salivary fluoride concentration for all 12 subjects (r = -0.68).



Another major factor which was anticipated might influence salivary fluoride clearance, and therefore the ambient fluoride concentrations, was the rinsing habits of individuals, either during or after tooth-brushing (Duckworth, Knoop & Stephen,1991; Sjögren & Birkhed,1993). However, in this group of subjects no relationship between their rinsing habits and their baseline salivary fluoride levels was demonstrated.

3.4.2 Salivary Fluoride Results.

The raw data from this experiment are tabulated in Appendix 3.5. The changes in ambient salivary fluoride concentration for all 12 subjects is presented graphically in Figures 3.2 - 3.6. Each of these figures shows a group of subjects who progressed through the fluoride dentifrices in the same order, e.g. subjects 1, 5 & 9 (Fig 3.2) used the 1500 ppm dentifrice followed by 500 ppm, non-F and 1000 ppm, whilst subjects 6 & 7 (Fig 3.5) used non-F, 1000 ppm, 1500 ppm and 500 ppm sequentially.

Close inspection of Figures 3.2 - 3.6 reveals a number of features. Firstly, there is wide individual variation, both in terms of the salivary fluoride concentration, and in the response to a change in dentifrice fluoride concentration. Some individuals, for example Subjects 11 and 12, seem to adapt to a different dentifrice within a few days, whilst others, such as Subjects 4 and 8, seem to demonstrate a cyclical pattern in response to the altered fluoride level. Secondly, the relationship between salivary flow rates and baseline fluoride concentration, apparent before the start of the experiment, appeared to be disrupted. Correlation coefficients were calculated for unstimulated salivary flow rates and the mean salivary fluoride values for each subject on each dentifrice. All the correlations were ≤ 0.44, indicating only a weak relationship between these parameters.

It should be noted that some subjects (2, 3, 10, 11 & 12) did not complete all four dentifrices. This occurred for a variety of reasons, namely the intrusion of holiday time into the experimental period (Subject 2), non-related illness (Subject 10), and a decision to

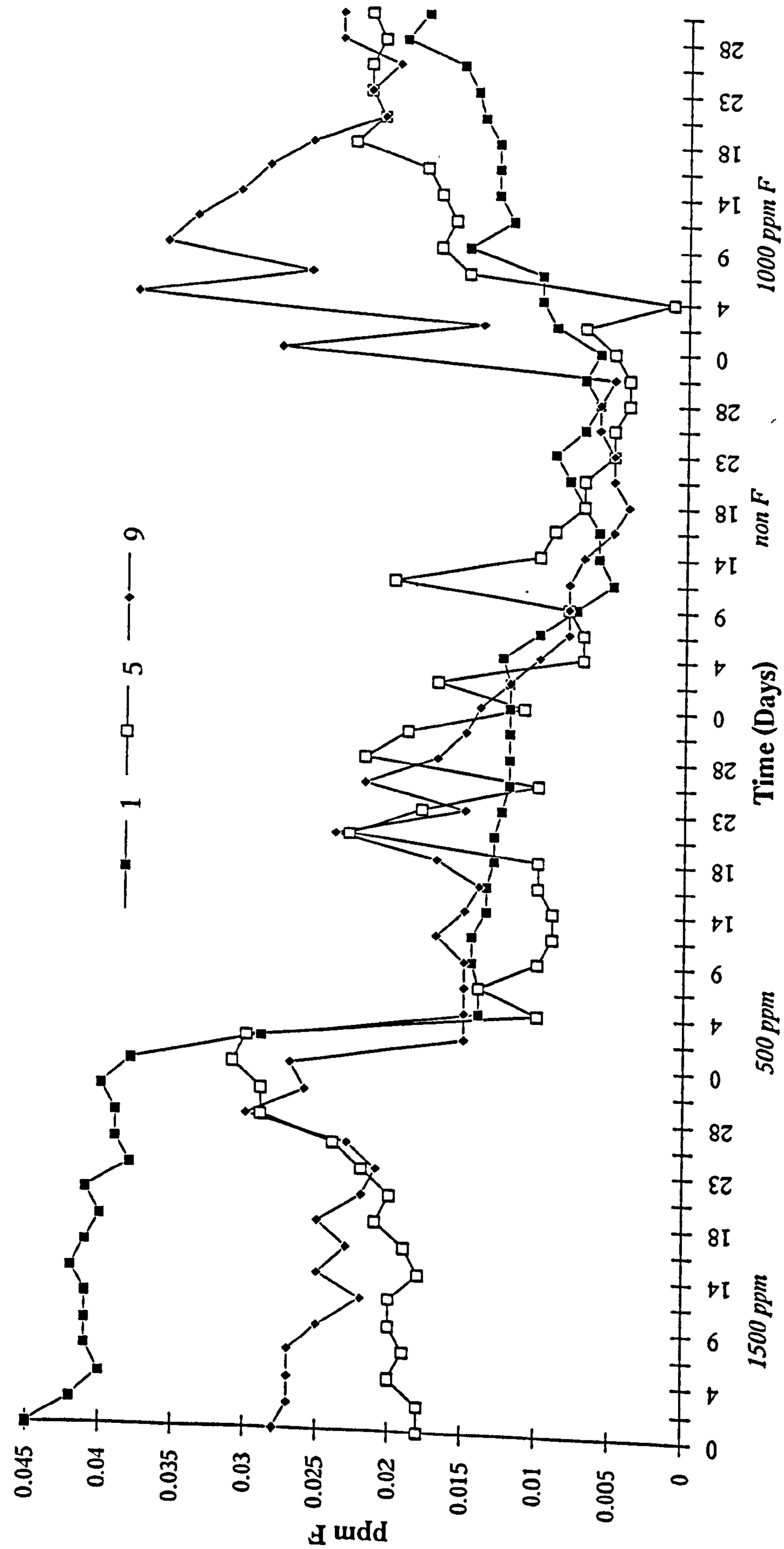


Figure 3.2 Whole salivary fluoride concentrations over time (days): Subjects 1, 5 & 9.

Figure 3.3 Whole salivary fluoride concentrations over time (days) : Subjects 2, 4 & 8.

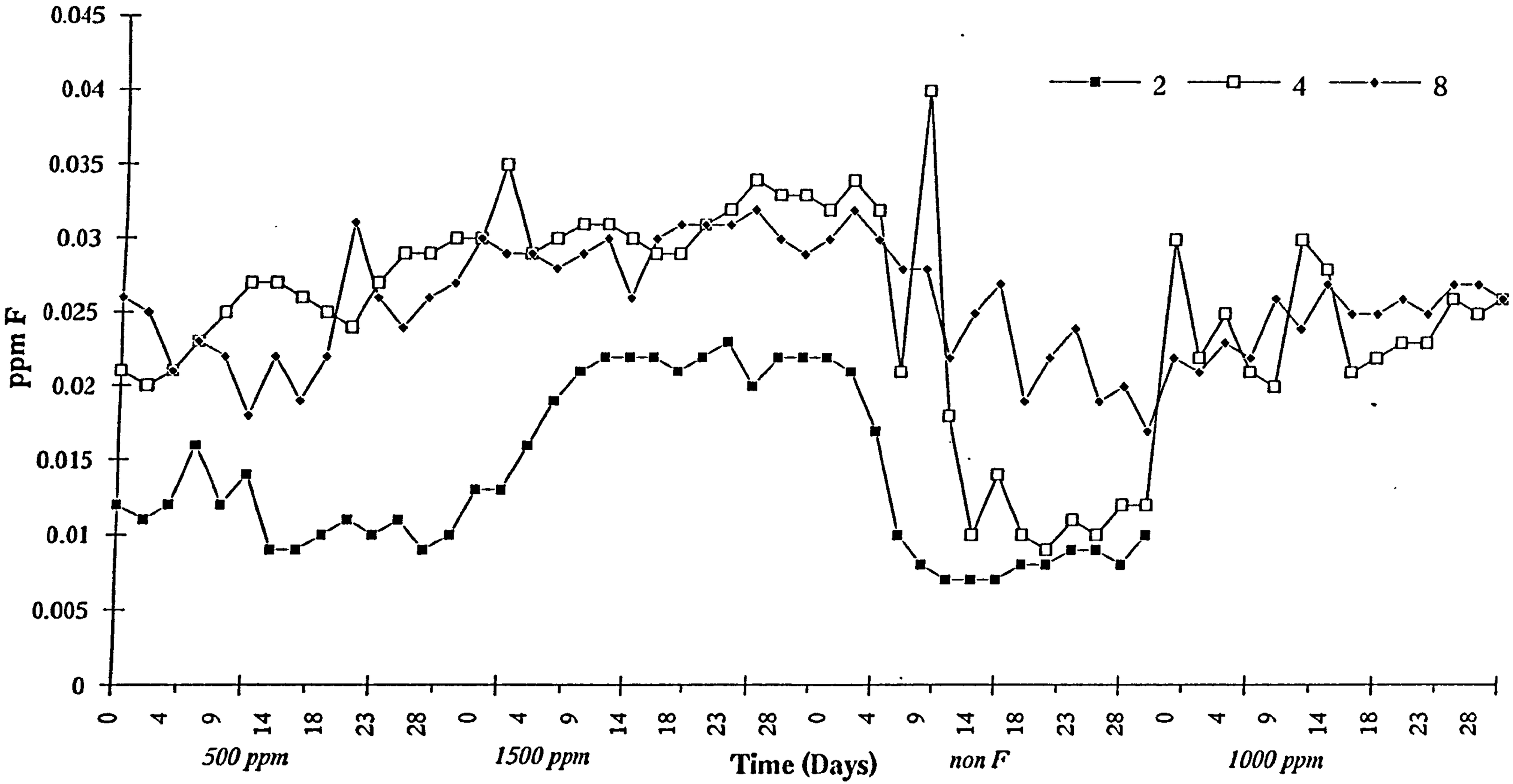


Figure 3.4 Whole salivary fluoride concentrations over time (days) : Subjects 3 & 10

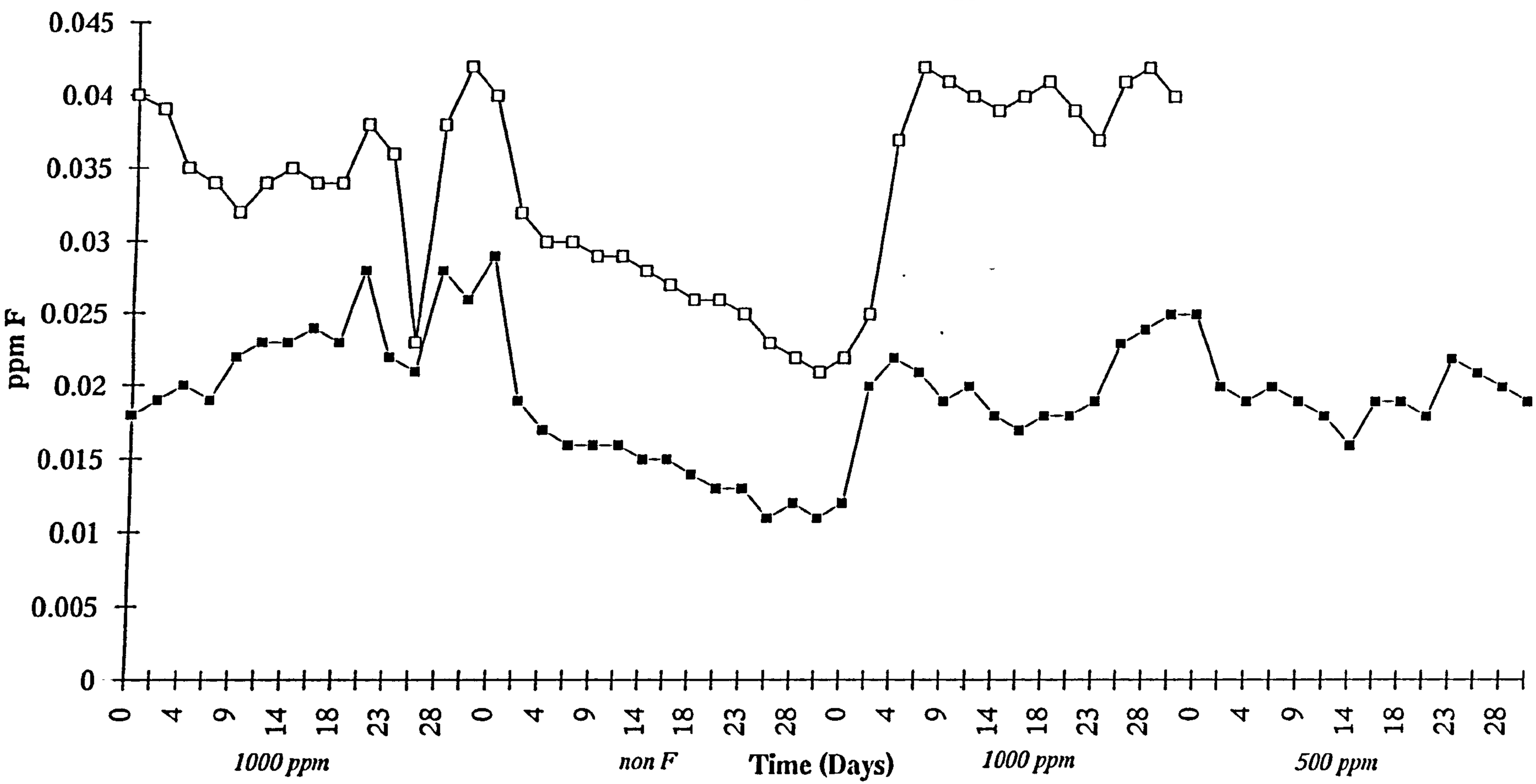


Figure 3.5 Whole salivary fluoride concentrations over time (days): Subjects 6 & 7.

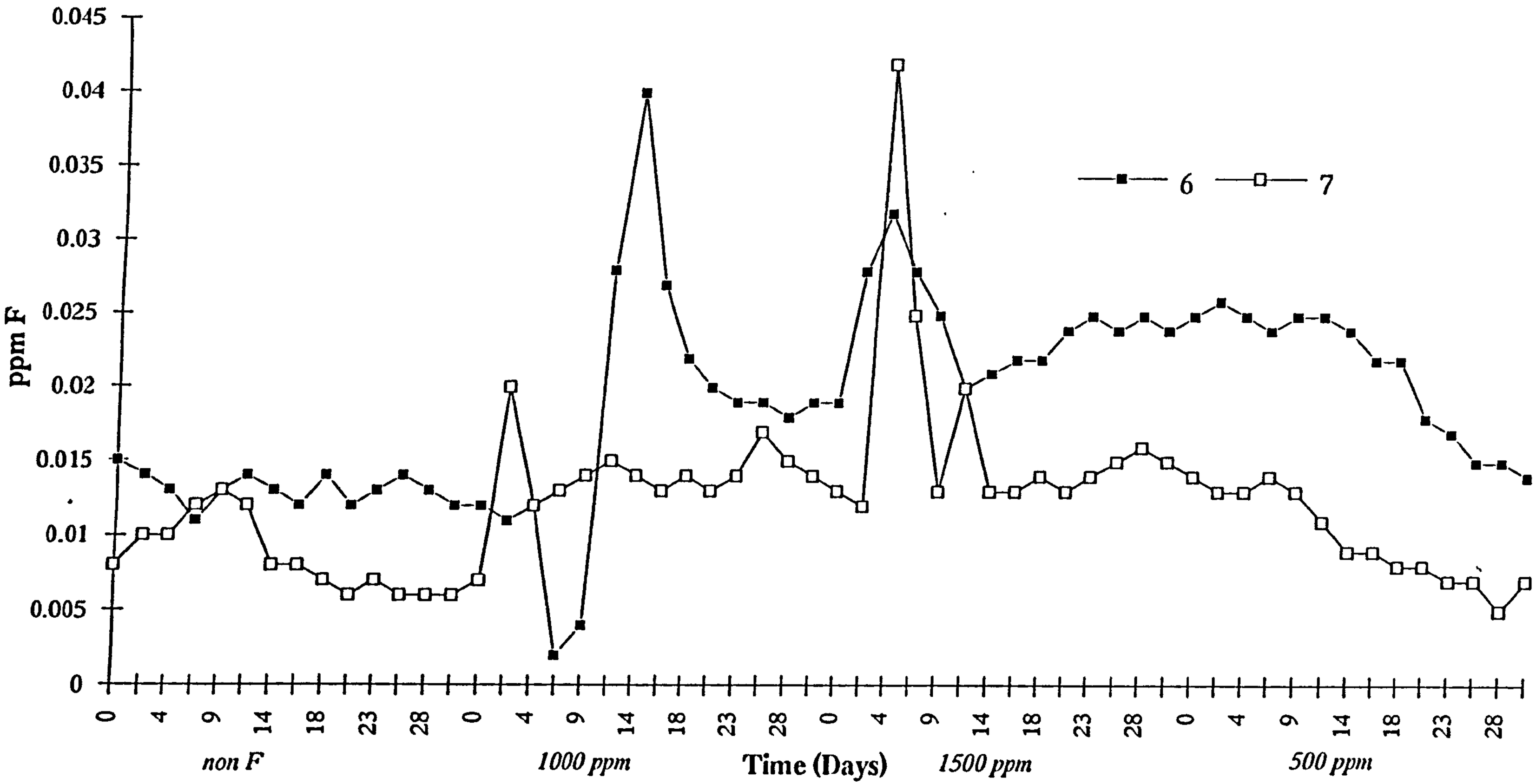


Figure 3.6 Whole salivary fluoride concentration over time (days): Subjects 11 & 12.

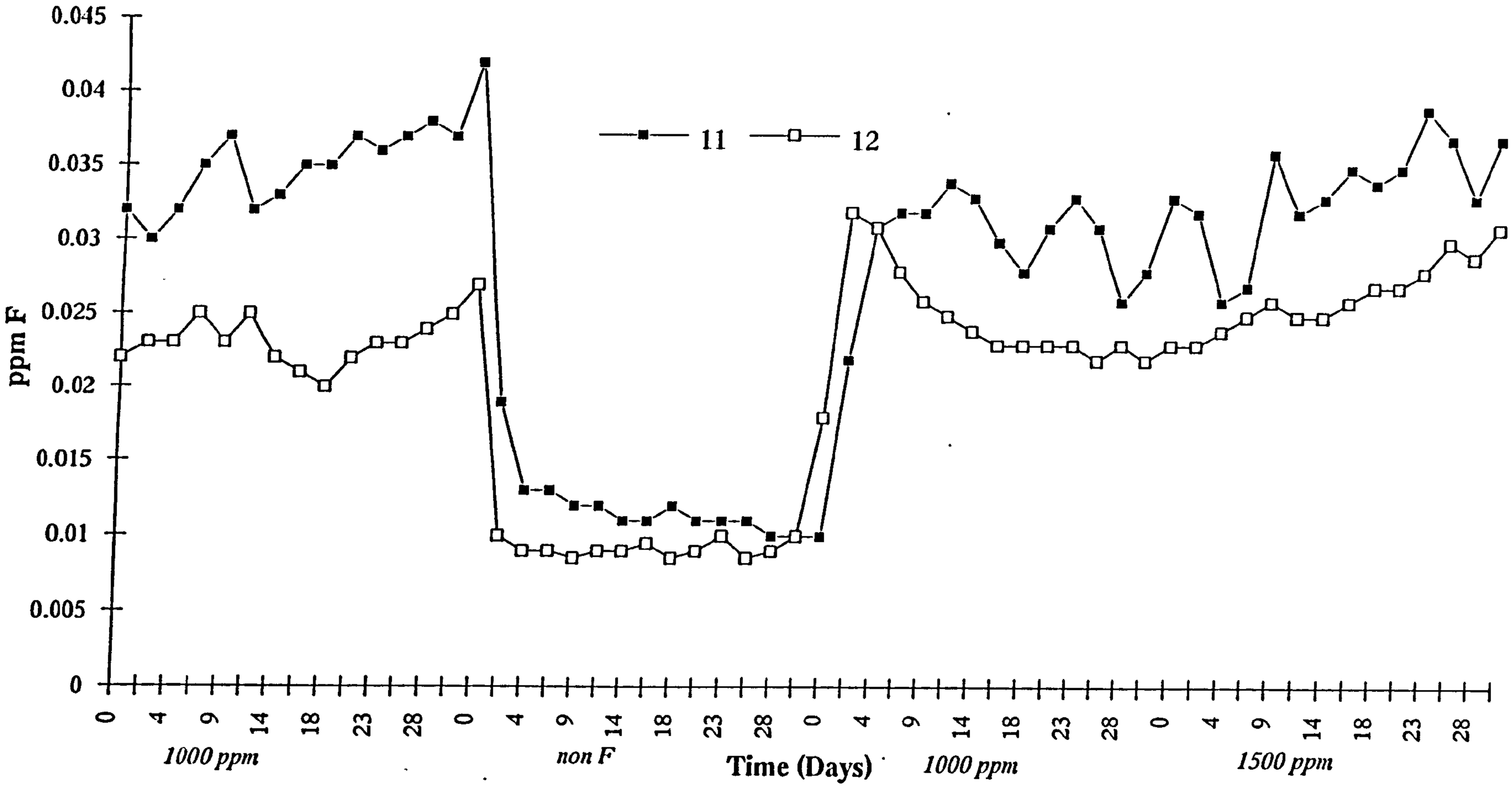
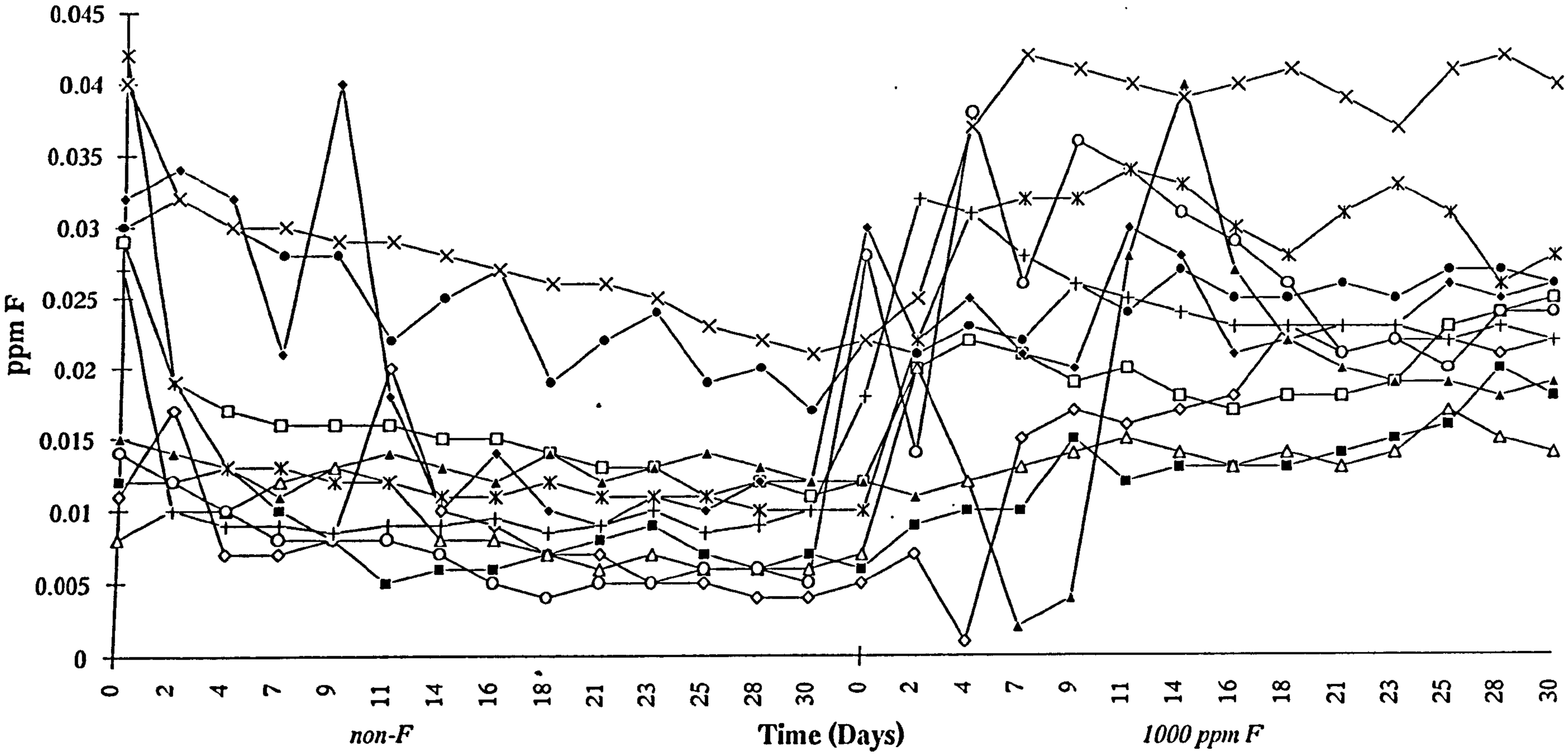


Figure 3.7 Whole salivary fluoride concentrations for all 12 subjects on non-F and 1000 ppm F dentifrices.



influence the order of the dentifrices (Subjects 3, 11 & 12). This decision was made after some early data collection, which indicated a significant amount of variability in the changeover period from one dentifrice to another. It was therefore decided to opt for a semi-random design, where all 12 subjects would use the 1000 ppm F dentifrice following the non-F dentifrice, regardless of the order in which they used the remaining test toothpastes. It was hoped this modification would allow a standard point of comparison between all 12 subjects (see Section 3.5 Discussion). The changes in salivary fluoride concentration for 11 of the 12 subjects (Subject 2 did not complete a 1000 ppm dentifrice) on the non-fluoride and 1000 ppm F dentifrices is presented in Figure 3.7. For nine of the subjects a steady decline of salivary fluoride concentration on the non-fluoridated paste is apparent, irrespective of the preceding dentifrice. However, one individual (Subject 4) recorded some very high values for the non-fluoridated dentifrice and, potentially, these data points may correspond to a protocol violation. Following the four week non-fluoridated period, all subjects recorded an increase in salivary fluoride levels on the introduction of the 1000 ppm F dentifrice. As can be seen from Figure 3.7 however, the response to this change was highly variable.

The mean values and standard error (S.E.) for salivary fluoride concentration for each volunteer on each dentifrice was calculated, and is presented in Table 3.2. The asterisks represent the missing data as discussed above. These data confirm the impression of a trend upwards of salivary fluoride with increasing dentifrice fluoride concentration, but again demonstrate the considerable inter-individual variation. It seems likely that some of the variability in this data set could have been eliminated if the subjects had progressed through the dentifrices in ascending order of fluoride concentrations, rather than in a semi-random manner, as was the case here.

The overall trend, however, is still apparent, as the combined data in Figure 3.8 and Table 3.3 demonstrate. The overall mean values for the 12 subjects on each dentifrice were: non F

- 0.014 ppm; 500 ppm F - 0.018 ppm; 1000 ppm F - 0.022 ppm and 1500 ppm F - 0.027 ppm. The variability observed in this pilot study indicated that protocol modifications were required and that statistical analysis of these data would be inappropriate.

3.5 Discussion.

This preliminary study answered a number of questions, and identified some weaknesses in the early experimental design. Firstly, the fluoride analytical equipment and the chosen technique were sufficiently sensitive to detect differences in salivary fluoride concentrations of the order of ± 0.005 ppm in the 0.01-0.05 ppm range. Below 0.01 ppm F the millivolt reading tended to drift for more prolonged periods, and the electrode response was less predictable. Therefore, 0.01 ppm F should be regarded as the limit of sensitivity of this technique.

Secondly, for any given group of individuals, there is likely to be a wide range of ambient salivary F concentrations. This must be multifactorial, and is likely to depend on such parameters as frequency and duration of toothbrushing; type and concentration of fluoride in the chosen dentifrice; dietary factors; salivary flow rate, and all those parameters identified as influencing the clearance of substances from the mouth (see Sections 3.1.2 and 3.1.3). More definitive results may have been forthcoming if the test dentifrices were used in ascending order of fluoride concentration, as this would help minimise the "carry-over" effect which was apparent in some subjects. It was decided, therefore, to repeat this experiment, with a modified study design and a larger number of volunteers.

Table 3.2 **Mean (S.E.) salivary fluoride levels (ppm F) versus dentifrice fluoride concentration for 12 volunteers (n=14).**

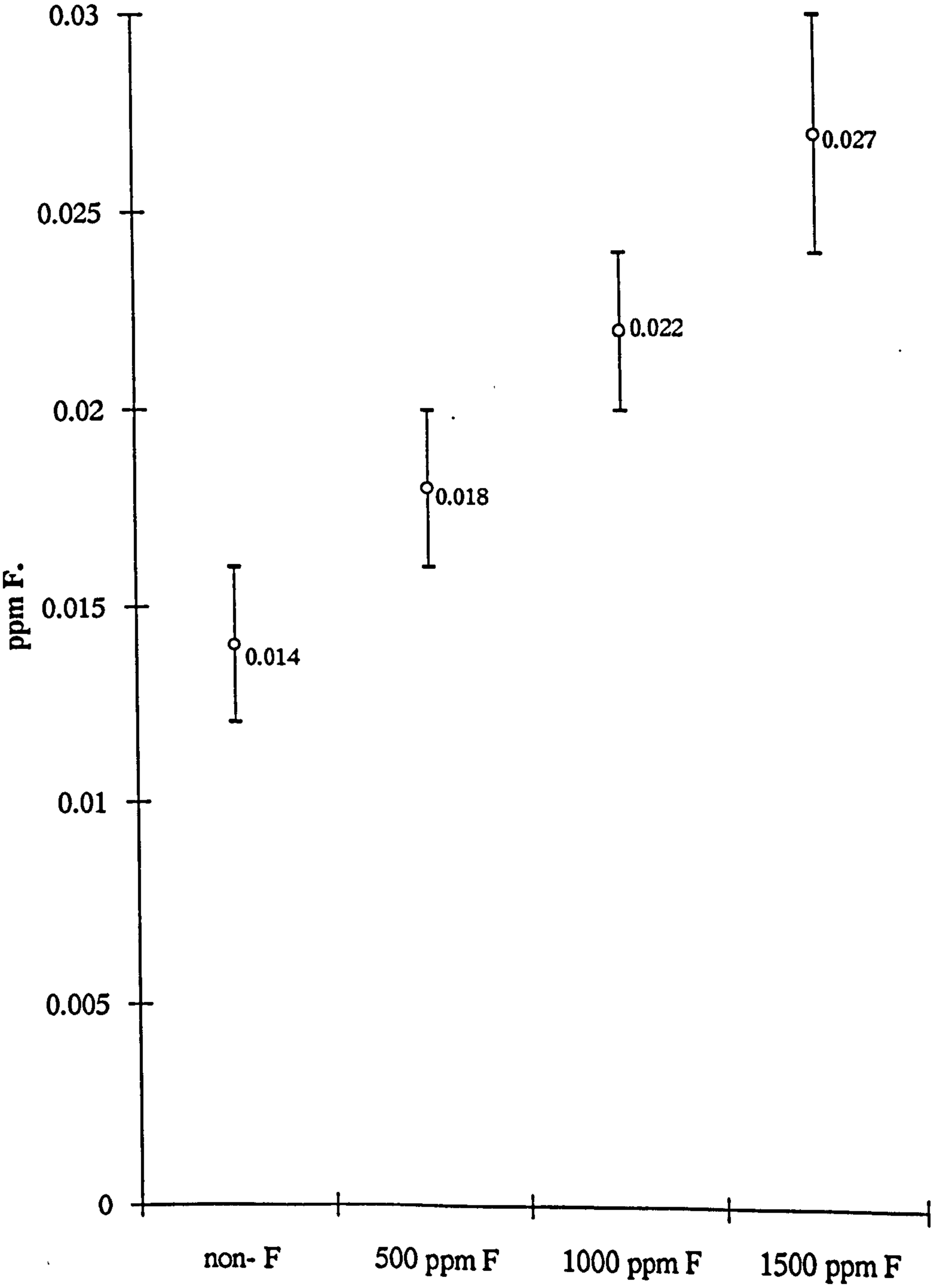
Subject	non-F dentifrice	S.E.	500 ppm F dentifrice	S.E.	1000 ppm F dentifrice	S.E.	1500 ppm F dentifrice	S.E.
1	0.008	0.001	0.016	0.002	0.013	0.001	0.041	0.001
2	0.011	0.001	0.011	0.001	*		0.020	0.001
3	0.015	0.001	0.020	0.001	0.020	0.001	*	
4	0.019	0.003	0.025	0.001	0.024	0.001	0.031	0.001
5	0.009	0.001	0.016	0.002	0.016	0.002	0.021	0.001
6	0.013	0.001	0.021	0.001	0.018	0.003	0.024	0.001
7	0.008	0.001	0.010	0.001	0.014	0.001	0.017	0.002
8	0.024	0.001	0.024	0.001	0.025	0.001	0.030	0.001
9	0.007	0.001	0.017	0.001	0.027	0.002	0.025	0.001
10	0.027	0.001	*		0.035	0.001	*	
11	0.014	0.002	*		0.028	0.002	0.033	0.001
12	0.010	0.001	*		0.024	0.001	0.026	0.001

*incomplete data

Table 3.3 **Overall mean salivary fluoride concentrations : combined data for all 12 subjects (ppm).**

	Mean	Minimum	Maximum	S.E.
non F	0.014	0.007	0.027	0.002
500 ppm	0.018	0.010	0.025	0.002
1000 ppm	0.022	0.013	0.035	0.002
1500 ppm	0.027	0.017	0.041	0.003

Figure 3.8 Mean (S.E.) whole salivary fluoride concentrations (ppm) versus dentifrice concentrations: all 12 subjects (n=144).



Chapter 4. Studies on the Influence of Dentifrice Fluoride Concentration on the Fluoride Concentration of Saliva, Plasma and Plaque.

4.1 Introduction.

The pharmacokinetics of fluoride has been the focus of investigations for many years (Ekstrand & Ehrnebo,1980; Whitford, Pashley & Pearson,1981; Ekstrand, Spak & Ehrnebo,1982; Whitford & Williams,1986; Whitford, Biles & Birdsong-Whitford,1991). However, as discussed in the preceding chapter, until recently the collective expert opinion suggested that either systemic uptake of fluoride during enamel maturation, or high concentrations of fluoride applied topically were required to achieve any cariostatic effect. In response to the accumulating evidence implying that, in fact, low concentrations of fluoride at the saliva:mineral interface were potentially cariostatic (Featherstone *et al.*1990), it was decided to repeat the experiment detailed in the previous chapter, to ascertain further how dentifrice fluoride concentration could influence the long-term low concentrations of fluoride in saliva. In addition to monitoring the fluoride concentration in mixed saliva, it was decided to monitor plaque fluoride levels as a further indicator of potential fluoride efficacy, and to monitor plasma fluoride levels, to determine if systemic fluoride levels were altered by changing the dentifrice fluoride concentration.

4.1.1 Plasma Fluoride & Fluorosis.

Plasma is the biological fluid into which, and from which fluoride must pass for its distribution elsewhere in the body, and for its elimination from the body. For these reasons it is referred to as the central compartment (see Section 1.3.8), and in this central compartment the fluoride ion is asymmetrically distributed between the plasma and the cellular component, such that the plasma concentration is approximately double that of the cells

(Whitford,1981).

There is a conflict of opinion as to the forms of fluoride which exist in plasma. On one hand, it has been claimed that as much as 80% of plasma fluoride may be bound to other macromolecules in blood (Guy, Taves & Brey,1976), whilst other studies (Chen *et al.*1956; Taves,1968; Ekstrand, Ericsson & Rosell,1977) utilising gel chromatography, have indicated that fluoride is not bound by plasma proteins or by any other constituent of plasma. Some of this discrepancy may be explained by the identification of a fluoride-containing contaminant, a surfactant molecule, which is widely used in commercially-available products. Currently, it is believed that the ionic fluoride, also called free fluoride or inorganic fluoride, is the phase considered significant in dentistry, medicine and public health and the fluoride-specific electrode is only sensitive to this fluoride component. In contrast, the biological significance of the bound fluoride, if it exists at all, has not been determined.

A wide range of 'normal' plasma levels is reported in the literature (Guy,1979), varying from 0.7 - 2.4 μM. Some of these disparate results may be due to the differing fasting conditions under which the samples were collected, as plasma fluoride levels are not homeostatically controlled, but rather rise and fall according to the pattern of fluoride intake. The earlier concept of fluoride homeostasis was based on findings showing that, among persons living in places with widely different water fluoride levels, plasma fluoride concentrations were similar. It was established subsequently that these findings were due to the use of inappropriate analytical techniques (Taves & Guy,1979). As plasma levels are not homeostatically controlled, there is no 'normal' physiological level. However, the plasma fluoride concentration expected in a healthy, fasted, long-term resident of a community with water fluoridated at 1ppm is approximately 1μM (0.019 ppm). Diurnal variations in plasma fluoride have also been noted (Ekstrand,1978), and are, in large part, due to circadian variations in the kinetics of the skeletal and renal dynamics of fluoride metabolism (Whitford *et al.*1983).

In addition to the level of chronic fluoride intake, plasma levels are influenced by the relative rates of bone accretion and dissolution, and by renal clearance rate of the ion. In the long term, there is a direct relationship between the concentrations of fluoride in bone and in plasma (Ericsson, Gydell & Hammarskiöld,1973), and as the fluoride content of bone tends to increase with age, there is also a direct relationship between age and fasting plasma fluoride (Parkins *et al.*1974).

There has been concern that there might exist a specific threshold for plasma fluoride that is associated with enamel fluorosis. It is known that the maximum concentration of plasma fluoride levels in individuals consuming water containing 1.2 ppm F may be as high as 2μM (0.038 ppm).The level of dental fluorosis in such populations is not clinically significant, and the conclusion is that plasma levels associated with fluorosis would have to be considerably higher than 2μM. In contrast, Ericsson *et al.* (1973) found plasma fluoride levels of 3-9 μM in residents of a community with drinking water containing 10 ppm F where fluorosis was endemic. Plasma levels of ≥10μM are unusual where the water fluoride concentration is in the range of 3-10 ppm (Ekstrand,1978), hence the conclusion that these small but recurring elevations in blood plasma (3-9 μM) during the period of enamel development are most commonly associated with cases of fluorosis. This conclusion is also supported by animal studies (Angmar-Månsson & Whitford,1982). Higher levels of fluorosis have been observed in young children ingesting fluoride tablets as a single daily dose when compared to a population ingesting the same level of fluoride in fluoridated water (Aasenden & Peebles,1974). This finding could be interpreted in terms of spiking of the plasma fluoride levels produced by a single high dose of fluoride which exceeds the fluorosis threshold. This type of evidence provides further support for the need to identify and characterise the effects of topical applications of fluoride, minimising the hazards of systemic uptake, and to establish the efficacy of much lower concentrations of fluoride than were previously considered.

4.1.2 Plaque Fluoride.

High levels of fluoride in plaque have been reported since the early 1960's (Hardwick,1961; Hardwick & Leach,1962) and these levels have been significantly higher than corresponding values reported for saliva. It has been concluded, therefore, that some mechanism exists which governs and maintains this concentration gradient.

The reported total concentration of fluoride in plaque ranges from 5-50 ppm (wet weight). Of this, approximately 1% is available as free fluoride ions, whilst 15-75% can be obtained in an ionised form by adding acid, neutral or basic buffers to the plaque residue, or by adding 0.5 M perchloric acid. The remainder of the plaque fluoride is present in a stable form and strong mineral acids and elevated temperatures are required for its release. It has, in fact, been noted that the firmly bound fluoride in plaque is more stable than fluorhydroxyapatite (Edgar,1980).

The fluoride found in plaque is likely to be derived from the diet and saliva (Jenkins & Edgar,1977), with tooth enamel considered to be an unlikely source. It has been suggested, in fact, that reverse exchange may occur, particularly on cervical enamel surfaces habitually covered by plaque where the fluoride concentration is high (Weatherell, Robinson & Hallsworth,1972; Weatherell *et al.*1977). If enamel fluoride was the source for plaque fluoride, regular toothbrushing with fluoride-free toothpaste, as in the data of Weatherell *et al.,* would have depleted fluoride in the cervical enamel. The reverse was found to be the case. Also, if the enamel surface is coated regularly with labile fluoride compounds, such as calcium fluoride (Rölla & Ögaard,1986), topical sources such as dentifrice fluoride may well contribute to the total fluoride content of plaque.

It is well established that calcium fluoride is formed on enamel, dentine and cementum, when these mineralised tissues are exposed to high concentrations of fluoride, such as in mouthrinses and dentifrices. It is also known that plaque fluid becomes supersaturated with

respect to calcium fluoride during such exposures, and that calcium fluoride is deposited in salivary sediments. The solubility characteristics of calcium fluoride are similar to that of fluoride in dental plaque, in that it is slightly soluble in water, in buffers and in 0.5 M perchloric acid, but dissolves extensively in strong mineral acids. The fact that calcium fluoride can be formed easily in the plaque matrix during topical use of fluoride, and that fluoride deposited in plaque has the solubility properties of calcium fluoride, indicates that calcium fluoride may be the major source of the fluoride in plaque. As stated above, considerable amounts of calcium fluoride are deposited on the oral mineralised tissues during topical application of fluoride products, and it can therefore, be extrapolated that the fluoride concentration of these products could influence the fluoride levels in plaque.

A variety of other sources of plaque fluoride have been suggested. Following intravenous infusion of ^{18}F, detectable levels of the tracer were found in the dental plaque of dogs, under conditions which excluded the access of saliva to the plaque (MacFadyen *et al.*1979). A gross calculation by these authors (1.9 mL crevicular fluid daily, at 1-2 μmol fluoride/L) suggests that 20-40 % of plaque fluoride may be of crevicular origin, although Edgar (1981) thought this unlikely because of the small volume of gingival crevicular fluid produced. If crevicular fluid is a source of plaque fluoride, it may be expected from the work of MacFadyen *et al.* (1979) and Whitford *et al.* (1981), that plasma peaks will be reflected in plaque and therefore a relationship between water fluoride and plaque fluoride might be expected, from both the topical effect during ingestion and a systemic effect via plasma F.

Fluoride in tea seems to have little effect on plaque fluoride, since Ophaug *et al.,* (1987) found no difference in plaque total fluoride, in subjects living in Minnesota (coffee as staple hot drink) and in Newcastle (tea), both of which used fluoridated water supplies for such preparations. However, drinking fluoridated water raises plasma fluoride concentration (see Section 4.1.2), and there appears to be a direct linear correlation between water fluoride concentration and plasma fluoride concentration (Taves & Guy,1979). The ratio of fluoride

in plasma and crevicular fluid is close to 1 (Whitford, Pashley & Pearson,1981), and plasma peaks may therefore be reflected in increased supply of fluoride to dental plaque by the crevicular route. Consistent with this, some authors have reported higher levels of total plaque fluoride where exposure to water fluoride was high (Agus *et al.*1976; Grobler, Reddy & van Wyk,1982; Nobre dos Santos & Cury,1988), although the scatter in such data clouds its interpretation.

Underlying much of the interest in determining plaque fluoride levels is the hypothesis that these concentrations may be correlated with caries prevalence, and that high plaque fluoride may be indicative of low caries risk. It is evident that such relationships are likely to be complicated, because of the time-scale of the caries process, the influence of dietary, microbiological and local factors, and the frequency and efficiency of plaque removal. Epidemiological evidence of associations between individual caries experience and plaque total fluoride content has been reported both in industrialised (Agus *et al.*1976) and in primitive populations (Schamschula *et al.*1978). Furthermore, Stiles *et al.* (1979), reported a direct relationship between mean group plaque fluoride and DMFS from three cohorts aged 12 to 18 years drinking water with < 5, 106, 212 μmol F/L (< 0.1, 2 and 4 ppm respectively), as well as a direct relationship between plaque fluoride and lifetime exposure to naturally fluoridated water. In addition, Agus *et al.* (1980) reported significant correlations between DMFS and bound plaque fluoride in both resting and fermenting plaque of unknown age from children aged 10-11 years. In contrast, Gaugler & Bruton (1982) measured bound fluoride in naval recruits on their first day of training, 70% of whom came from areas receiving fluoridated water. No significant difference in plaque fluoride was found between those coming from fluoridated and non-fluoridated areas. However, the caries-free recruits had significantly higher mean plaque fluoride levels than the caries-active subjects, which may have been a reflection of more frequent toothbrushing with fluoridated dentifrices, and may support further the hypothesis that plaque fluoride is influenced

significantly by the composition and frequency of application of topical fluoride agents.

4.2 Study Aims & Introduction.

This aims of this extended study were, therefore, as follows:

1. to establish the relationship between dentifrice fluoride concentration and ambient (i.e. 4 hours post-brushing) mixed salivary fluoride levels, which was suggested by the previous experiment,

2. to monitor the fluoride concentration in plaque in a sub-group of volunteers, and determine if there was any relationship between plaque fluoride and dentifrice fluoride, and/or between plaque fluoride and salivary fluoride

and

3. to monitor the fluoride concentration in plasma in the same sub-group, and identify any relationship with dentifrice fluoride concentration, with salivary fluoride concentration and/or with plaque fluoride concentration.

4.2.1 Study Population.

A number of the volunteers from the previous experiment (Chapter 3) were willing to undertake this second study (subject numbers 1, 3, 4, 5, 6, 7, 9 & 10), and an additional 12 subjects were recruited from the staff of Glasgow University Dental School, giving a total of 20. There were 10 males and 10 females, with a mean age of 33.4 years (S.D. 8.9), all subjects were dentate and routinely used a fluoridated dentifrice. Each subject underwent a full clinical examination using a plane mirror (No. 5) and a CPITN (Community Periodontal Index of Treatment Needs) probe, including DMFS, calculus score (Volpe, Manhold & Hazen, 1965) and oral hygiene assessment (Greene & Vermillion,1960). Each subject also completed a questionnaire in relation to their normal toothbrushing/oral hygiene procedures,

including their favoured brand of toothpaste. Paraffin-wax stimulated salivary flow rates were measured, along with salivary buffering capacity *(Dentobuff, Orion Diagnostica,* Finland).

4.2.2 Volunteer Protocol.

All 20 volunteers were provided with a sodium fluoride dentifrice, containing a silica abrasive, from the same batch as the previous study *(Unilever Research,* U.K.). These dentifrices contained 0.22 ppm F, 500 ppm F, 1000 ppm F and 1500 ppm F respectively, and were identically packaged and flavoured. A detailed protocol, outlining the requirements for the study, was issued to each volunteer, and is included as Appendix 3.2. All volunteers agreed to refrain from using any other fluoride-containing product for the duration of the study (4 months), to brush with the test dentifrices twice daily, and to refrain from rinsing during toothbrushing. They all remained blind to the dentifrice fluoride concentrations throughout the experimental period.

4.2.3 Experimental Protocol Outline.

Pre-experiment saliva samples were collected from all volunteers, and similarly, samples of plaque and plasma were collected from those who had agreed (n=10) to this additional testing. The four test dentifrices were issued in order of increasing fluoride concentration, with each dentifrice being used throughout a four week period. Saliva samples were collected on alternate days at mid-day, in order to maximise the time since last toothbrushing and last food intake, and were stored at -16°C until required for analysis. Plaque and plasma samples were collected once per week, also at mid-day, from the subgroup of ten volunteers and were similarly stored until required. The details of the collection and analytical procedures are provided in the following sections.

4.3 Materials & Methods.

4.3.1 Saliva.

Mixed, unstimulated saliva samples were collected from all 20 subjects on alternate days, by drooling collected saliva into a bijou bottles over a 5 minute period, as detailed in the previous chapter (Section 3.3.3). This procedure resulted in variable amounts of saliva being collected, depending on the individual's unstimulated salivary flow rate, with a minimum of approximately 2 g being required. Once the sample had been collected, the bottles were sealed, placed in re-sealable polythene bags, and stored in a freezer at -16°C. At approximately weekly intervals, all the previous week's saliva samples (n=60) were removed from the freezer and allowed to return to room temperature (~18°C) over a period of 2 hours. During this time, the *Orion 901 Ionalyzer* and the fluoride specific electrode/reference electrode were calibrated according to the procedure detailed in Appendix 3.4 (Indirect method). The saliva samples were vortex mixed for 1 minute, to redistribute the high molecular weight material, then 0.5 ml of saliva was pipetted into a microsample dish. An equal volume of Total Ionic Strength Adjusting Buffer (*TISAB, Orion Res. Inc. U.S.A.)* was added to the sample, which was agitated continuously by magnetic stirring. The electrodes were then introduced carefully into the sample, and a 10 minute equilibration time was set. The mV reading was recorded at this time, the electrodes rinsed in de-ionised water and blotted dry, and the procedure repeated for the next sample. Calibration of the system was repeated every 3 hours of measuring, and the entire weeks' samples were measured over a 2 day period (approximately 12 hours of measuring time). The mV readings for the standard solutions were then fed into a computer software programme, *Fluofit*, written "in house" by Dr R Strang, which used a least squares technique to fit a polynomial curve to the logarithmic transformation of the standard solution concentration, and calculate the sample concentrations from this curve.

4.3.2 Plasma.

In the sub-group of 10 volunteers, who participated in the protocol, capillary blood was drawn at the weekly visit, using a modification of a micromethod first described by Ekstrand (1977). A fingertip haemorrhage was initiated using a *Monolet* lancet (*Sherwood Medical,* St. Louis, Missouri, U.S.A.), in a *Monojector* lancet device (*Sherwood Medical)* as shown in Figure 4.1 and 4.2. Pressure was applied to maintain a small blood flow, which was collected directly into a 0.75 mL *Microvette* vial (*Sarstedt,* Germany) impregnated previously with an EDTA anticoagulant (Figure 4.3). Once 0.75 ml was collected, the vial was sealed, placed directly into a centrifuge (*Centaur,* M.S.E.) and spun at 3500 rpm for 5 minutes. The plasma was then pipetted into a fresh Eppendorf tube, sealed and stored at -16 °C until required for analysis.

Fluoride analysis was undertaken using the same equipment as described in Appendix 3.4. Plasma samples were allowed to come to room temperature before the individual Eppendorf tubes for each subject were mixed thoroughly on the vibromixer to redistribute any high molecular weight material. A micropipette was used to transfer 500 µls of plasma to a microsampling dish, and an equal volume of low-level *TISAB* was added. The fluoride concentration was determined using the equipment and techniques described previously.

4.3.3 Plaque.

In the sub-group of 10 individuals participating in the protocol, weekly samples were collected from the buccal and lingual surfaces of the posterior teeth (≥ 2 mgs wet weight) using a sterile metal spatula, placed in a pre-weighed sterile Eppendorf tube and sealed. The plaque weight was then determined using a *Sartorius MC1* balance (*Sartorius,* Gottingen, Germany), which has a resolution of 0.1 mg. All samples were handled with gloved hands to avoid cross-infection and contamination. Immediately after weighing, the samples were frozen at -16°C until required for analysis.

Figure 4.1 **Photograph of a *Monolet* lancet (*Sherwood Medical,* U.S.A.) for initiating fingertip haemorrhage.**

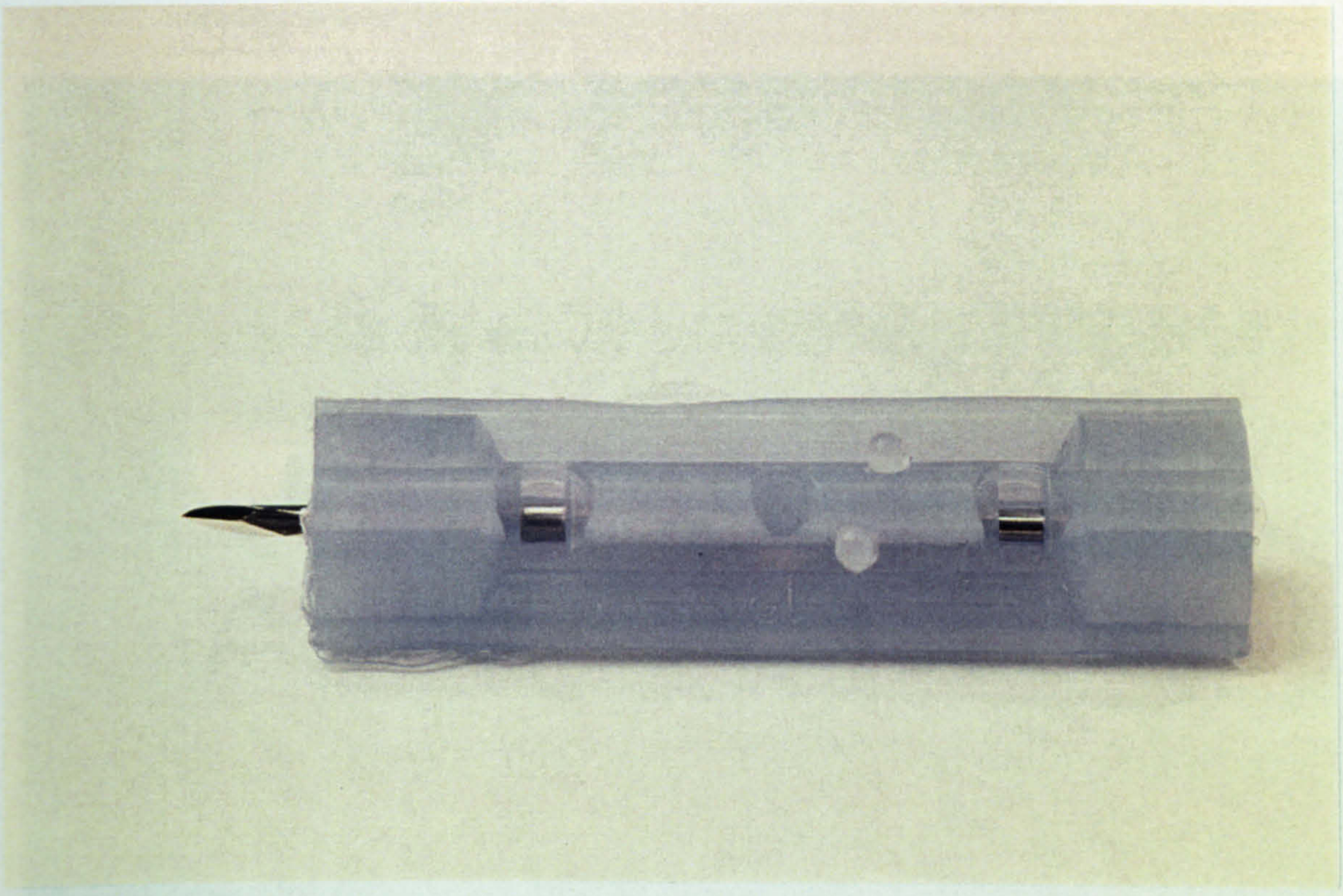


Figure 4.2 **Photograph of the *Monojector* lancet spring-loaded device (*Sherwood Medical,* U.S.A.) for firing *Monoject* lancets to initiate fingertip haemorrhage.**

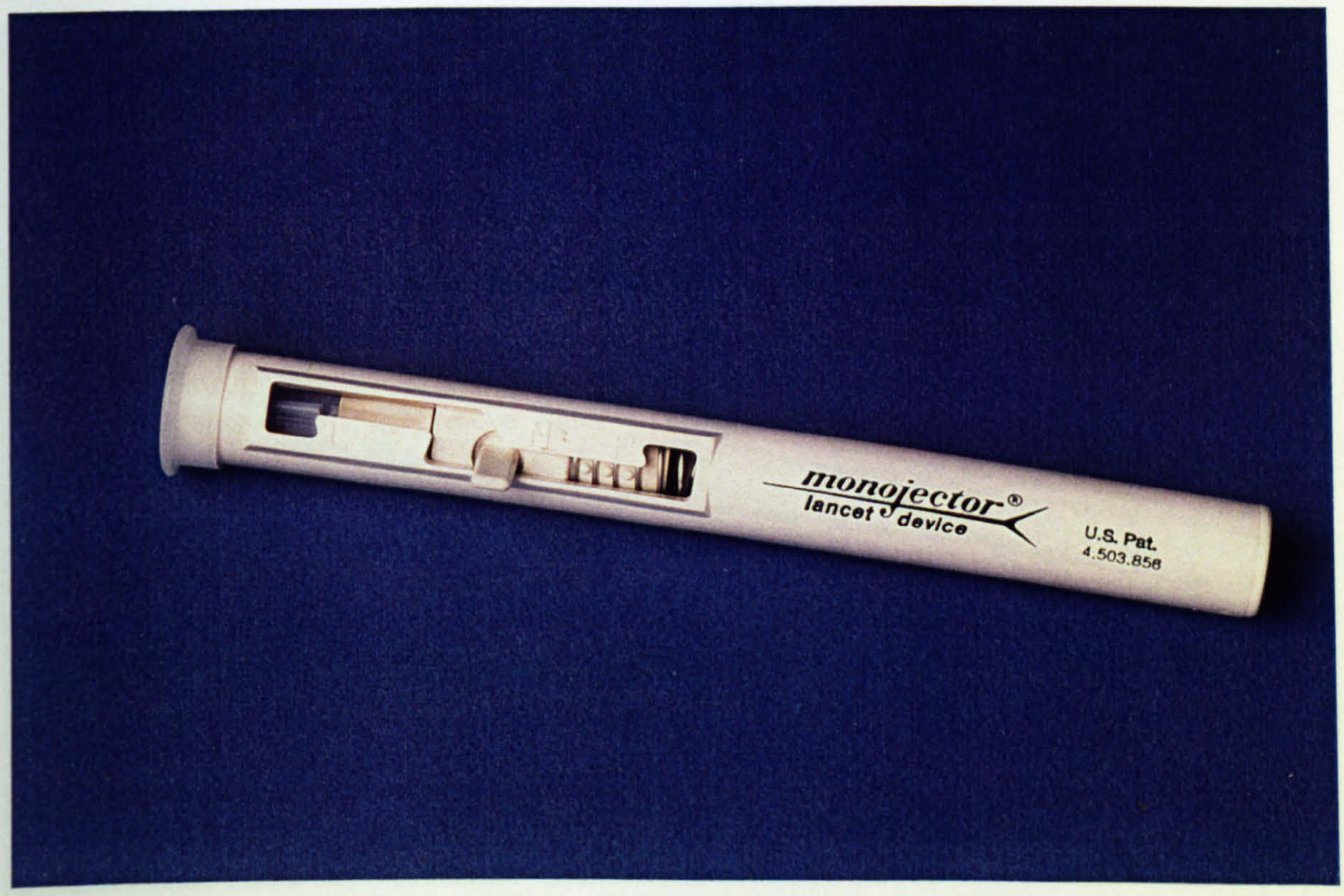
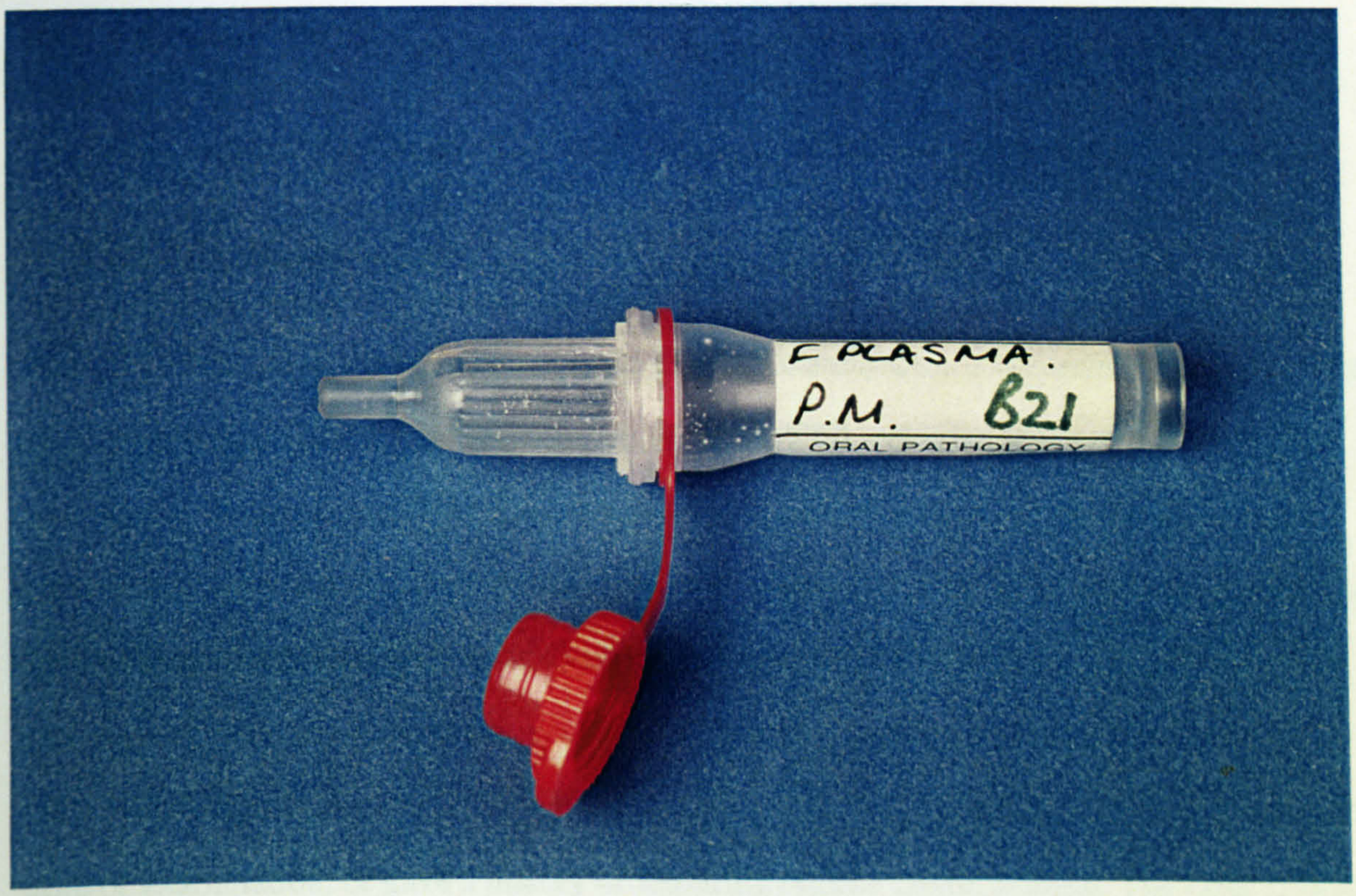


Figure 4.3 **Photograph of 0.75 mL *Microvette* vial (*Sarstedt,* Germany) impregnated with EDTA, for the direct collection of blood from a fingertip haemorrhage.**



Fluoride analysis was undertaken using the same equipment as described in Appendix 3.4. Plaque samples were allowed to come to room temperature before adding 350µl of deionised water. The individual Eppendorf tubes containing plaque and water for each subject were mixed thoroughly on the vibromixer to produce a homogenous suspension suitable for analysis. The samples were then mixed with equal quantities of low level *TISAB,* and the fluoride concentration determined in the manner described previously. The concentration of fluoride in the sample was expressed in parts per million fluoride per milligram wet weight of plaque.

4.4 Results.

The saliva sample raw data relating to this study is included as Appendix 4.1.

4.4.1 Baseline Data.

The data collected at the pre-experimental clinical examination is recorded in Table 4.1, and includes the age, DMFT, DMFS, decayed surfaces (DS), calculus score and oral hygiene assessment. It can be seen that there was a considerable variation in the previous caries experience of the study group, as evidenced by the range in DMFS (range 8 - 65), although the decayed component contributed very little to the DMFS total. Thus, in general this group had a high caries experience, but virtually all carious surfaces had been restored or extracted. Calculus was present in 9 of 20 subjects, but only 2 had significant deposits at the time of examination, or gave a history of requiring regular dental scaling. The Oral Hygiene Index (O.H.I.) was calculated as a maximum possible '3' on each of 6 teeth i.e. maximum total of 18, and this total was divided by 6 (teeth), to give an overall 'mouth' score (maximum = 3). The oral hygiene assessment scores ranged from 0 - 0.33, indicating that, in general, the group had reasonable oral hygiene.

The data collected via the volunteer questionnaire (Appendix 3.6) is presented in Table 4.2,

Table 4.1 Baseline demographic data for 20 subjects.

Volunteer	Age	DMFT	DMFS	DS	Calculus*	O.H.I.**
1	32	17	53	-	-	0.22
2	27	16	60	-	-	0.17
3	36	13	31	-	-	0.06
4	42	22	63	-	5	0.27
5	26	6	8	-	2	0.11
6	45	21	65	-	-	0.22
7	28	18	35	-	-	0
8	28	7	9	-	-	0.11
9	22	11	20	-	2	0.17
10	33	15	35	-	4	0.33
11	28	13	40	2	-	0.27
12	20	12	30	-	14	0.17
13	55	16	41	-	4	0.27
14	22	6	12	-	-	0.5
15	31	17	40	-	-	0.33
16	41	21	40	1	4	0.22
17	42	17	35	-	2	0
18	42	15	21	1	18	0.27
19	40	19	47	-	-	0
20	31	12	23	-	-	0

*Volpe-Manhold

**Greene & Vermillion

Table 4.2 Subject responses to tooth-brushing questionnaire.

Volunteer	Daily brushing	Time (mins)	Dentifrice	Rinse/spit	Tea*	Other
1	3	1.5	McLeans	Y/Y	1	-
2	2	2.5	Colgate	Y/Y	4	Floss
3	1	0.5	Colgate	Y/Y	0	-
4	4	2	Mentadent P	N/Y	0	Woodsticks
5	3	2	Sensodyne	Y/Y	7	Floss
6	3	1	Colgate	Y/N	4	Floss
7	3	2	Mentadent P	N/Y	1	Floss
8	2	1	Colgate	N/Y	2	-
9	5	1.5	Colgate	Y/Y	3	Floss
10	4	3	Colgate	Y/Y	3	Floss
11	3	3	Crest	Y/Y	3	-
12	2	5	Colgate	Y/Y	3	Floss
13	2	2	Sensodyne	Y/Y	1	Floss
14	1	2	Gibbs SR	Y/Y	2	Electric toothbrush
15	2	1	Aquafresh	Y/N	4	-
16	2	1	Colgate	Y/N	2	-
17	2	2.5	Colgate	Y/Y	6	Floss
18	2	1	Crest Anti-tartar	Y/Y	0	Floss
19	2	2	Crest	N/Y	4	Floss
20	3	3.5	Sensodyne	Y/N	0	Floss

*Units/day (standard tea-cup)

and represents the toothbrushing habits of the subjects prior to starting this study. Of the 20 volunteers, 9 claimed to routinely clean their teeth more than twice per day, which may be a reflection of the generally increased level of dental awareness within a group of dental school staff. In addition, the reported use of additional oral hygiene measures, (14 out of 20), is surprisingly high, although the volunteers were not asked how often they employed these additional measures.

Rinsing out, particularly with a glass of water, and frequent spitting during brushing have been shown to influence the retention of fluoride in the mouth after toothbrushing (Duckworth & Morgan,1992; Sjögren & Birkhed, 1993). The normal habits of the volunteers in terms of rinsing and spitting was therefore recorded (Table 4.2), although rinsing was prohibited for the duration of the experimental period. Finally, average daily tea consumption was recorded, as the only likely source of significant quantities of fluoride in the typical West of Scotland diet.

The buffering capacity and stimulated salivary flow rates are presented in Table 4.3, with considerable variation occurring in both these parameters : stimulated flow rate range 0.55 - 2.70 g/min; buffering capacity range 3.96 - 7.35 (final pH). Correlation co-efficients were calculated for these two parameters with DMFS, and with each other, and these are presented in Table 4.4. A weak positive correlation was identified between flow rate and buffering capacity (0.38), whilst a weak negative correlation existed between DMFS and buffering capacity, which are directional as would be anticipated. The relationship between flow rate and DMFS is slightly more difficult to interpret. A high salivary flow rate is obviously advantageous in terms of flushing fermentable carbohydrate and bacterial metabolic products, such as lactic acid, out of the oral cavity. However, this same high flow rate will also increase the rate of fluoride clearance from the mouth, and therefore also have a potentially negative impact on fluoride efficacy. In this instance, the correlation coefficient for flow rate and DMFS was positive, at 0.48.

Table 4.3 **Baseline stimulated salivary flow rate and salivary buffering capacity for 20 subjects.**

Volunteer	Stimulated Salivary Flow (g/min)	Stimulated Buffering Capacity
1	2.15	6.32
2	1.71	5.31
3	1.23	4.90
4	1.64	3.20
5	1.78	6.94
6	1.98	6.02
7	1.02	4.63
8	1.18	4.50
9	0.94	4.53
10	0.68	5.14
11	0.55	3.96
12	0.56	4.02
13	0.95	5.37
14	0.66	7.35
15	1.42	4.42
16	1.80	4.86
17	0.90	5.68
18	1.20	4.37
19	2.70	7.32
20	1.20	6.81

Table 4.4 **Correlations between DMFS, stimulated salivary flow rates and salivary buffering capacity for 20 subjects.**

	DMFS	Buffering capacity	Stimulated flow rate
DMFS		-0.18	0.48
Buffering capacity	-0.18		0.38
Stimulated flow rate	0.48	0.38	

4.4.2 Saliva Results.

The salivary fluoride data are presented in Tables 4.5 - 4.14, and Figures 4.4, 4.5 & 4.6. Data in Table 4.5 show the mean fluoride level of all the salivary samples (n=12) for each volunteer, on each dentifrice i.e. one fluoride value for each volunteer on each paste, (Subject 10 was unable to complete the fourth leg of the study due to an unrelated illness), and Figure 4.4 is a graphical representation of the same data. The overall mean (S.E.) values for all 20 subjects combined (Table 4.6), with each dentifrice were : 0.01 (0.001), 0.017 (0.001), 0.023 (0.001) and 0.041 (0.004) for the non F, 500 ppm F, 1000 ppm F and the 1500 ppm F dentifrices, respectively (all values ppm F). Figure 4.5 is a graphical representation of these data.

In order to assess the potential impact of a carry-over effect, the mean salivary fluoride concentrations for the first two weeks on each dentifrice (weeks 1+2) were compared with the mean values for the second two weeks (weeks 3+4), using a Two-sample T-test and the results of this comparison are presented in Table 4.7. As a result of the differences identified here, particularly for the non-F and 500 ppm dentifrices ($p=0.023$, $p=0.002$), additional comparisons were made, also using a Two-sample T-test, between the mean salivary fluoride values for weeks 1+2 and all four weeks on each dentifrice (weeks 1-4), and also for the second two weeks (weeks 3+4) and all four weeks (weeks1-4). The results of these comparisons are presented in Tables 4.8 and 4.9, and a summary of all the T-test comparisons is provided in Table 4.10. Furthermore, Figure 4.6 illustrates the same data in graphical format.

Subsequently, the data set was subjected to two types of Analysis of Variance (ANOVA). Firstly, ANOVA for the effect of "duration of dentifrice usage" was undertaken, for each dentifrice concentration, and the results are included in Table 4.11.1, Table 4.11.2 and Table 4.11.3. This analysis confirmed the earlier T-tests, and demonstrated a significant difference

for salivary fluoride levels for the non F paste (weeks 1+2 v weeks 3+4, p=0.001; weeks 1+2 v weeks 1-4, p= 0.001; weeks 3+4 v weeks1-4, p=0.001) and the 500 ppm F paste (weeks 1+2 v weeks 3+4, p<0.001; weeks 1+2 v weeks 1-4, p<0.001; weeks 3+4 v weeks 1-4, p<0.001). No significant differences were identified from the same comparisons undertaken for the 1000 ppm F and the 1500 ppm F dentifrices (p≥0.59). A summary of the ANOVA results is provided in Table 4.12.

The second ANOVA was undertaken on the complete data set i.e. weeks 1-4, for all subjects on each paste, to identify the effects of dentifrice fluoride concentration. Volunteer effects were also analysed at this time. The results of this second ANOVA are included in Table 4.13. Highly significant differences of salivary fluoride concentration (p<0.001) with dentifrice fluoride concentration were identified, in addition to highly significant volunteer effects (p=0.001).

Finally, linear regression analysis of salivary fluoride concentration on dentifrice fluoride concentration (all four dentifrices) for each volunteer was undertaken, and is presented in Table 4.14, including the intercept, the slope of the regression line and the significance level.

Table 4.5 **Mean salivary fluoride levels (n = 12) for each of 20 subjects for each dentifrice fluoride concentration (ppm F).**

Volunteer	non - F dentifrice	500 ppm F dentifrice	1000 F ppm dentifrice	1500 ppm F dentifrice
1	0.010	0.016	0.023	0.033
2	0.014	0.025	0.024	0.045
3	0.008	0.011	0.018	0.022
4	0.006	0.009	0.011	0.020
5	0.012	0.018	0.018	0.030
6	0.013	0.015	0.018	0.030
7	0.014	0.021	0.040	0.045
8	0.006	0.014	0.019	0.023
9	0.011	0.011	0.016	0.017
10	0.013	0.022	0.035	*
11	0.008	0.013	0.015	0.034
12	0.010	0.017	0.025	0.050
13	0.020	0.017	0.025	0.065
14	0.011	0.017	0.030	0.045
15	0.011	0.013	0.018	0.035
16	0.013	0.019	0.021	0.053
17	0.009	0.017	0.018	0.032
18	0.011	0.014	0.022	0.037
19	0.007	0.020	0.028	0.060
20	0.011	0.025	0.029	0.073

* = missing data

Figure 4.4 Mean (n=12) salivary fluoride concentration (ppm) for each of 20 subjects versus dentifrice fluoride concentration.

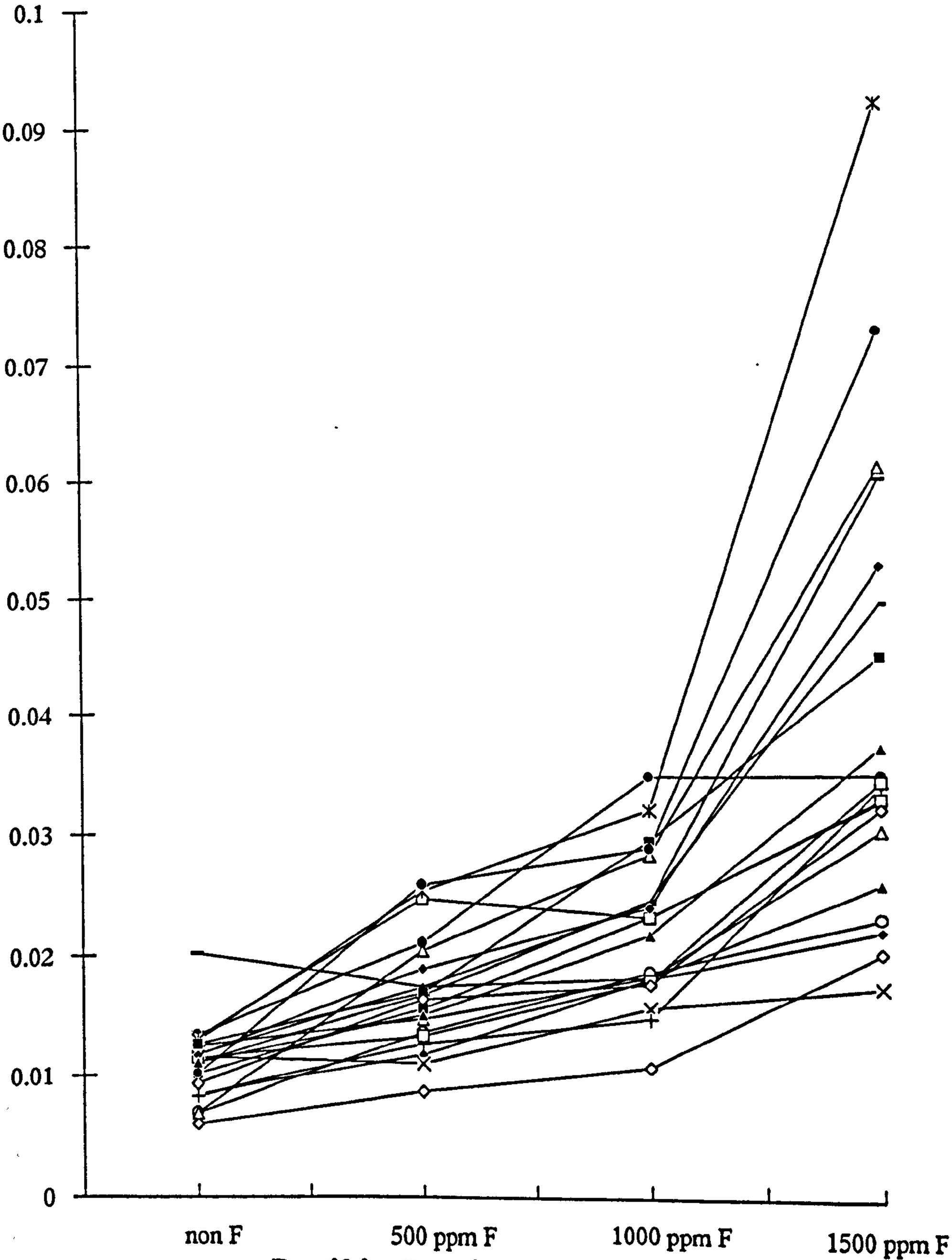


Table 4.6 **Overall mean (S.E.) and 95% confidence intervals of salivary fluoride versus dentifrice concentration (ppm)**

Dentifrice	mean	n	S.E.	95% C.I.
non F	0.011	235	0.001	0.009, 0.012
500 ppm F	0.017	219	0.001	0.015, 0.019
1000 ppm F	0.023	215	0.001	0.019, 0.025
1500 ppm F	0.041	232	0.004	0.032, 0.049

Figure 4.5 Mean (95% C.I.) salivary fluoride concentration (ppm) for all subjects.

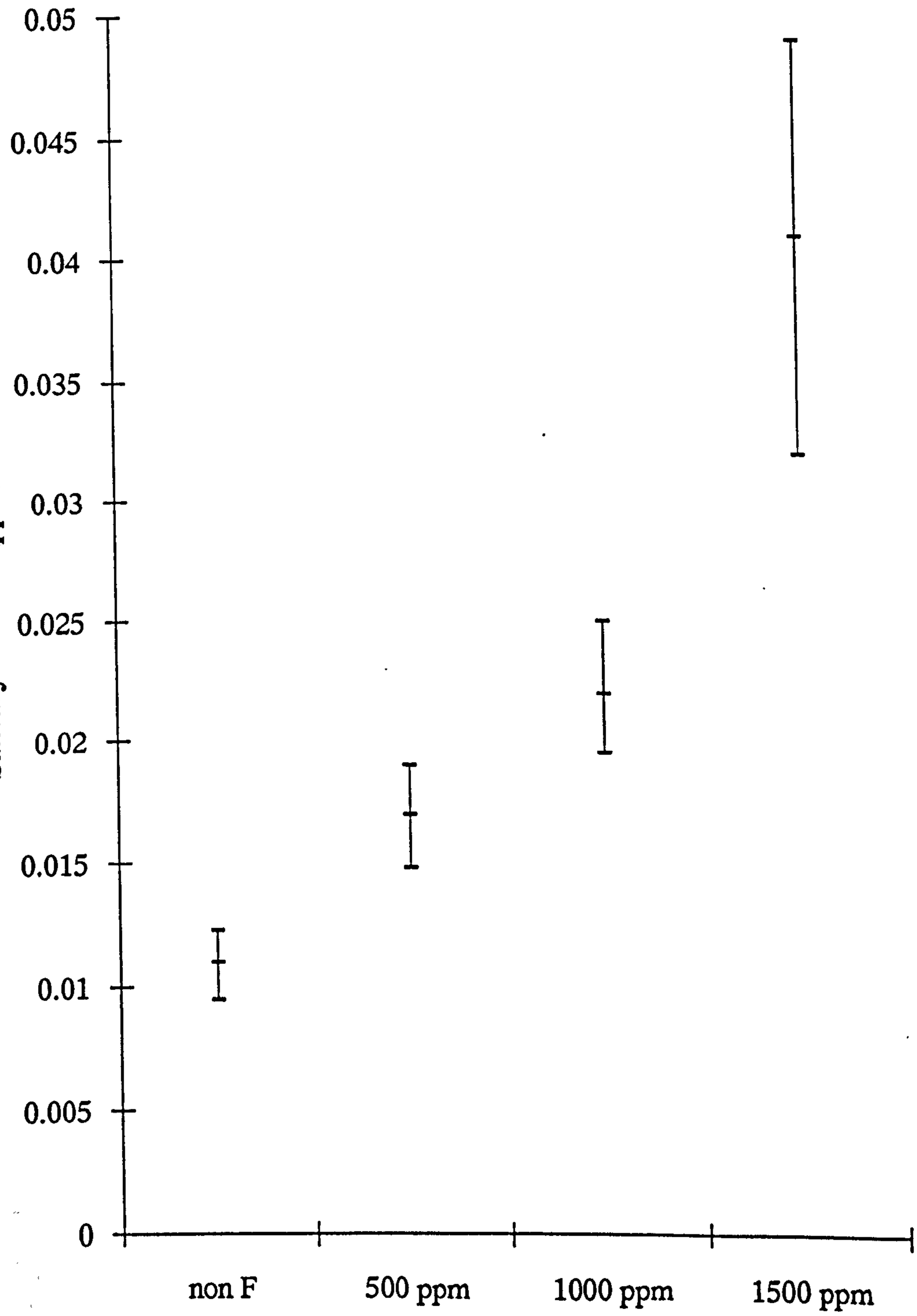


Table 4.7 **Comparison of mean (S.E.) for weeks 1+2 and weeks 3+4 salivary fluoride concentration versus dentifrice concentration (ppm F) *Two-sample T-test*.**

Dentifrice	mean (n = 20)		S.E.		p value
	week 1+2	week 3+4	week 1+2	week 3+4	
non F	0.010	0.012	0.001	0.001	0.023
500 ppm F	0.014	0.020	0.001	0.001	0.002
1000 ppm F	0.022	0.022	0.001	0.002	0.84
1500 ppm F	0.041	0.041	0.005	0.004	0.94

Table 4.8 **Comparison of the mean (S.E.) for weeks 1+2 and weeks 1 to 4 salivary fluoride concentration versus dentifrice concentration (ppm F)** ***Two-sample T-test.***

Dentifrice	mean (n = 20)		S.E.		p value
	weeks 1+2	weeks 1-4	weeks 1+2	weeks 1-4	
non F	0.010	0.011	0.001	0.001	0.25
500 ppm F	0.014	0.017	0.001	0.001	0.08
1000 ppm F	0.022	0.022	0.001	0.002	0.88
1500 ppm F	0.041	0.041	0.005	0.004	0.97

Table 4.9 **Comparison of the mean (S.E.) for weeks 3+4 and weeks 1 to 4 salivary fluoride concentration versus dentifrice concentration (ppm F)** ***Two-sample T-test.***

Dentifrice	mean (n=20)		S.E.		p value
	week 3+4	week 1-4	week 3+4	week 1-4	
non F	0.012	0.011	0.001	0.001	0.19
500 ppm F	0.020	0.017	0.001	0.001	0.10
1000 ppm F	0.022	0.022	0.002	0.001	0.95
1500 ppm F	0.041	0.041	0.004	0.004	0.97

Table 4.10 Summary of the comparisons of weeks 1+2 v weeks 3+4 v weeks 1 to 4. *Two sample T-test.*

Dentifrice and Time.	non F w1+2	non-F w3+4	non F w1-4	500 ppm w1+2	500 ppm w3+4	500 ppm w1-4	1000 ppm w1+2	1000 ppm w3+4	1000 ppm w1-4	1500 ppm w1+2	1500 ppm w3+4	1500 ppm w1-4
non F w1+2	—	*	NS									
non F w3+4	*	—	NS									
non F w1-4	NS	NS	—									
500 ppm w1+2				—	***	NS						
500 ppm w3+4				***	—	NS						
500 ppm w1-4				NS	NS	—						
1000 ppm w1+2							—	NS	NS			
1000 ppm w3+4							NS	—	NS			
1000 ppm w1-4							NS	NS	—			
1500 ppm w1+2										—	NS	NS
1500 ppm w3+4										NS	—	NS
1500 ppm w1-4										NS	NS	—

NS = not significant ($p \geq 0.05$)

* = $0.01 < p < 0.05$

** = $0.005 < p < 0.01$

*** = $p < 0.005$

Figure 4.6 Mean (95% C.I.) salivary fluoride concentration (ppm) for all subjects (n=20) for weeks 1+2 (1), weeks 3+4 (2) and weeks 1 to 4 (3) for each dentifrice fluoride concentration.

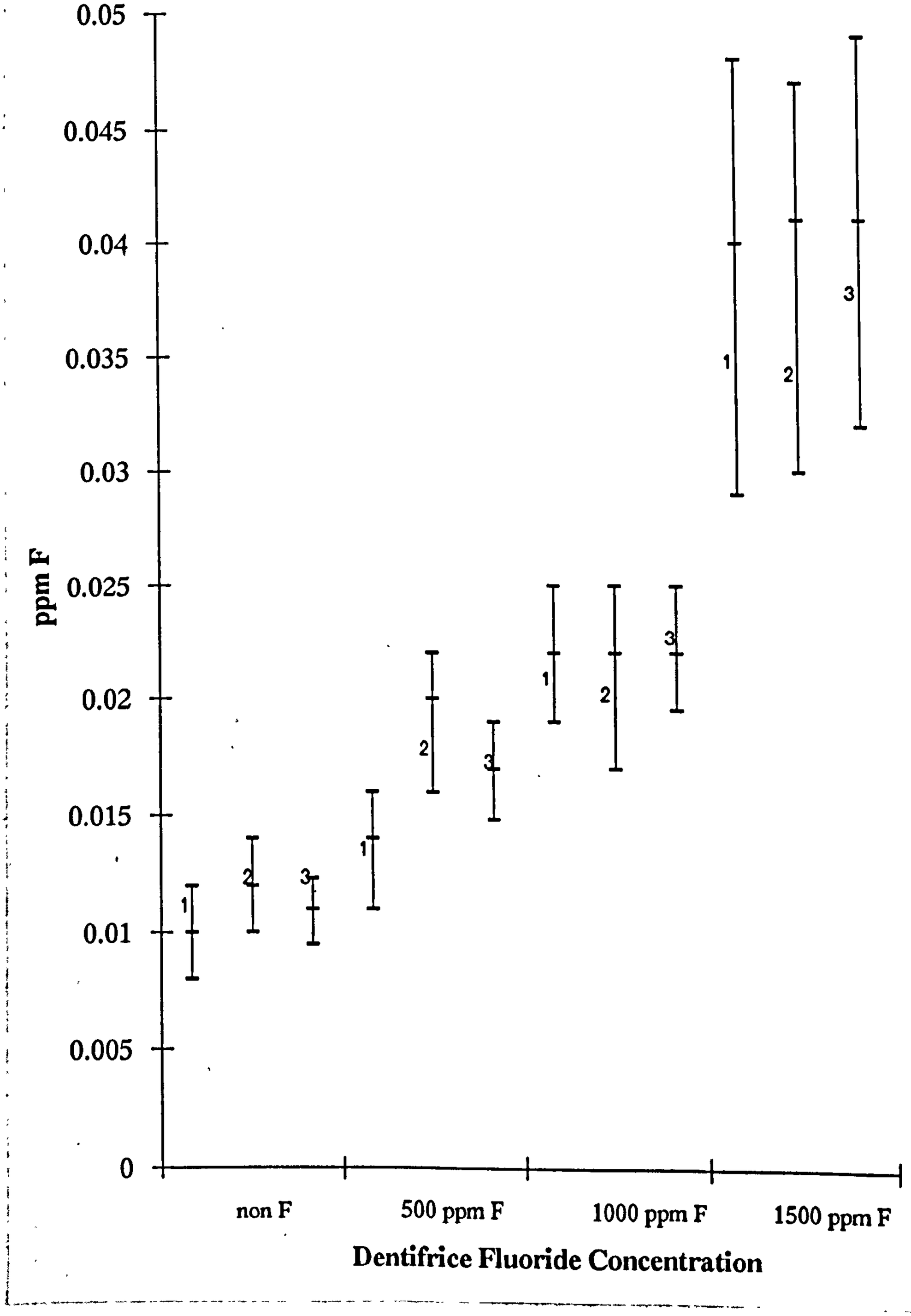


Table 4.11.1 Salivary fluoride concentration: Analysis of variance with time and volunteer effects (non F and 500 ppm F dentifrices) for Weeks 1+2 and Weeks 3+4.

Dentifrice	Variable	Degrees of Freedom	Sum of Squares	Mean Square	F Statistic	p value
non F	Time*	1	0.0000654	0.0000654	15.66	0.001
	Volunteer**	19	0.0003602	0.0000189	4.54	0.001
	Error	19	0.0000794	0.0000042		
	Total	39	0.0005051			
500 ppm F	Time*	1	0.0002976	0.0002976	35.22	< 0.001
	Volunteer**	19	0.0008587	0.0000452	5.35	<0.001
	Error	19	0.0001606	0.0000084		
	Total	39	0.0013169			

*Time = comparison of Weeks 1 +2 and Weeks 3+4
**Volunteer = Individuals salivary fluoride concentration

Table 4.11.1(cont) **Salivary fluoride concentration: Analysis of variance with time and volunteer effects (1000 ppm F and 1500 ppm F dentifrices) for Weeks 1+2 and Weeks 3+4.**

Dentifrice	Variable	Degrees of Freedom	Sum of Squares	Mean Square	F Statistic	p value
1000 ppm F	Time*	1	0.0000019	0.0000019	0.15	0.707
	Volunteer**	19	0.0015489	0.0000815	6.23	<0.001
	Error	19	0.0002487	0.0000131		
	Total	39	0.0017995			
1500 ppm F	Time*	1	0.0000022	0.0000022	0.06	0.809
	Volunteer**	19	0.0141526	0.0007449	20.60	<0.001
	Error	19	0.0006871	0.0000362		
	Total	39	0.0148418			

*Time = comparison of Weeks 1+2 and Weeks 3+4
**Volunteer = Individuals salivary fluoride concentration.

Table 4.11.2 Salivary fluoride concentration: Analysis of variance with time and volunteer effects (non F and 500 ppm F dentifrices) for Weeks 1+2 and Weeks 1to 4.

Dentifrice	Variable	Degrees of Freedom	Sum of Squares	Mean Square	F Statistic	p value
non F	Time*	1	0.0000166	0.0000166	15.72	0.001
	Volunteer**	19	0.0004319	0.0000227	21.51	<0.001
	Error	19	0.0000201	0.0000011		
	Total	39	0.0004686			
500 ppm F	Time*	1	0.0000759	0.0000759	37.54	<0.001
	Volunteer**	19	0.0008666	0.0000456	22.53	<0.001
	Error	19	0.0000384	0.0000020		
	Total	39	0.0009811			

*Time = comparison of Weeks 1 +2 and Weeks 1-4
**Volunteer = Individuals salivary fluoride concentration

Table 4.11.2 (cont) Salivary fluoride concentration: Analysis of variance with time and volunteer effects (1000 ppm F and 1500 ppm F dentifrices) for Weeks 1+2 and Weeks 1 to 4.

Dentifrice	Variable	Degrees of Freedom	Sum of Squares	Mean Square	F Statistic	p value
1000 ppm F	Time*	1	0.0000008	0.0000008	0.29	0.594
	Volunteer**	19	0.0012176	0.0000641	24.22	<0.001
	Error	19	0.0000503	0.0000027		
	Total	39	0.0012686			
1500 ppm F	Time*	1	0.0000007	0.0000007	0.07	0.798
	Volunteer**	19	0.0151365	0.0007966	76.33	<0.001
	Error	19	0.0001983	0.0000104		
	Total	39	0.0153355			

*Time = comparison of Weeks 1+2 and Weeks 1-4
**Volunteer = Individuals salivary fluoride concentration.

Table 4.11.3 Salivary fluoride concentration: Analysis of variance with time and volunteer effects (non F and 500 ppm F dentifrices) for Weeks 3+4 and Weeks 1 to 4.

Dentifrice	Variable	Degrees of Freedom	Sum of Squares	Mean Square	F Statistic	p value
non F	Time*	1	0.0000161	0.0000161	15.56	0.001
	Volunteer**	19	0.0003295	0.0000173	16.75	<0.001
	Error	19	0.0000196	0.0000010		
	Total	39	0.0003653			
500 ppm F	Time*	1	0.0000728	0.0000728	31.35	<0.001
	Volunteer**	19	0.0009253	0.0000487	20.96	<0.001
	Error	19	0.0000442	0.0000023		
	Total	39	0.0010423			

*Time = comparison of Weeks 3+4 and Weeks 1-4
**Volunteer = Individuals salivary fluoride concentration

Table 4.11.3 (cont) **Salivary fluoride concentration: Analysis of variance with time and volunteer effects (1000 ppm F and 1500 ppm F dentifrices) for Weeks 3+4 and Weeks 1 to 4.**

Dentifrice	Variable	Degrees of Freedom	Sum of Squares	Mean Square	F Statistic	p value
1000 ppm F	Time*	1	0.00000024	0.00000024	0.06	0.808
	Volunteer**	19	0.0019290	0.0001015	24.81	<0.001
	Error	19	0.0000777	0.0000041		
	Total	39	0.0020070			
1500 ppm F	Time*	1	0.00000041	0.00000041	0.05	0.822
	Volunteer**	19	0.01343585	0.0007071	90.60	<0.001
	Error	19	0.00014829	0.0000078		
	Total	39	0.01358455			

*Time = comparison of Weeks 3+4 and Weeks 1-4
**Volunteer = Individuals salivary fluoride concentration.

Table 4.12 **Summary of comparisons of weeks 1+2 versus weeks 3+4 v weeks 1 to 4.*Analysis of Variance.***

Dentifrice and Time.	non F w 1+2	non-F w3+4	non F w1-4	500 ppm w1+2	500 ppm w3+4	500 ppm w1-4	1000 ppm w1+2	1000 ppm w3+4	1000 ppm w1-4	1500 ppm w1+2	1500 ppm w3+4	1500 ppm w1-4
non F w1+2	—	**	***									
non F w3+4	**	—	***									
non F w1-4	***	***	—									
500 ppm w1+2				—	***	***						
500 ppm w3+4				***	—	***						
500 ppm w1-4				***	***	—						
1000 ppm w1+2							—	NS	NS			
1000 ppm w3+4							NS	—	NS			
1000 ppm w1-4							NS	NS	—			
1500 ppm w1+2										—	NS	NS
1500 ppm w3+4										NS	—	NS
1500 ppm w1-4										NS	NS	—

NS = not significant ($p \geq 0.05$)

* = $0.01 < p < 0.05$

** = $0.005 < p < 0.01$

*** = $p < 0.005$

Table 4.13 **Salivary fluoride concentration: Analysis of Variance with dentifrice fluoride concentration and volunteer effects.**

Variable	Degrees of Freedom	Sum of Squares	Mean Square	F Statistic	p value
Dentifrice Fluoride Concentration	3	0.00998	0.00333	44.77	<0.001
Volunteer	19	0.00709	0.00021	20.86	0.001
Error	57	0.00429	0.00075		
Total	79	0.01836			

Table 4.14 **Slope of the linear regression line for salivary fluoride concentration versus dentifrice fluoride concentration for each volunteer.**

Volunteer	Intercept	Slope (x 10^{-4})	p value
1	0.91	0.15	0.01
2	1.32	0.18	0.08
3	0.74	0.10	0.01
4	0.49	0.09	0.05
5	1.17	0.01	0.08
6	1.09	0.11	0.08
7	1.32	0.22	0.03
8	0.71	0.11	0.01
9	1.02	0.05	0.06
10	0.14	0.55	0.10
11	0.55	0.16	0.09
12	0.63	0.26	0.05
13	1.03	0.29	0.18
14	0.85	0.23	0.02
15	0.79	0.15	0.08
16	0.82	0.24	0.12
17	0.85	0.14	0.05
18	0.81	0.17	0.04
19	0.37	0.33	0.04
20	0.60	0.38	0.08

4.4.3 Plasma Results.

The mean plasma fluoride levels for each volunteer, on each dentifrice, are set out in Table 4.15, and presented graphically in Figure 4.7. One volunteer, Subject 6, had consistently higher plasma fluoride values than the remainder of the group.

In addition, the overall mean (n=40) plasma values for the subset of ten volunteers on each dentifrice are presented in Table 4.16, along with the standard error and 95% confidence intervals. In Figure 4.8, the same data are presented in a graphical format.

Finally, a two-way ANOVA was undertaken to identify the influence of dentifrice fluoride concentration on plasma fluoride concentration, and to identify any potential volunteer effects. The results of this ANOVA are included in Table 4.17, which shows highly significant differences for plasma fluoride with dentifrice fluoride ($p<0.001$), in addition to significant volunteer effects ($p<0.001$).

Table 4.15 **Mean plasma fluoride levels for ten subjects (n = 4) for each dentifrice concentration (ppm F).**

Volunteer	non-F dentifrice	500 ppm F dentifrice	1000 ppm F dentifrice	1500 ppm F dentifrice
1	0.013	0.018	0.018	0.020
2	0.017	0.021	0.024	0.027
3	0.013	0.018	0.024	0.031
4	0.011	0.020	0.021	0.022
5	0.013	0.015	0.019	0.020
6	0.021	0.028	0.041	0.045
7	0.010	0.017	0.019	0.019
8	0.017	0.020	0.022	0.026
9	0.010	0.014	0.019	0.021
10	0.011	0.015	0.018	0.022

Figure 4.7 Mean plasma fluoride concentration (ppm) for each of 10 subjects (n=4) versus dentifrice fluoride concentration.

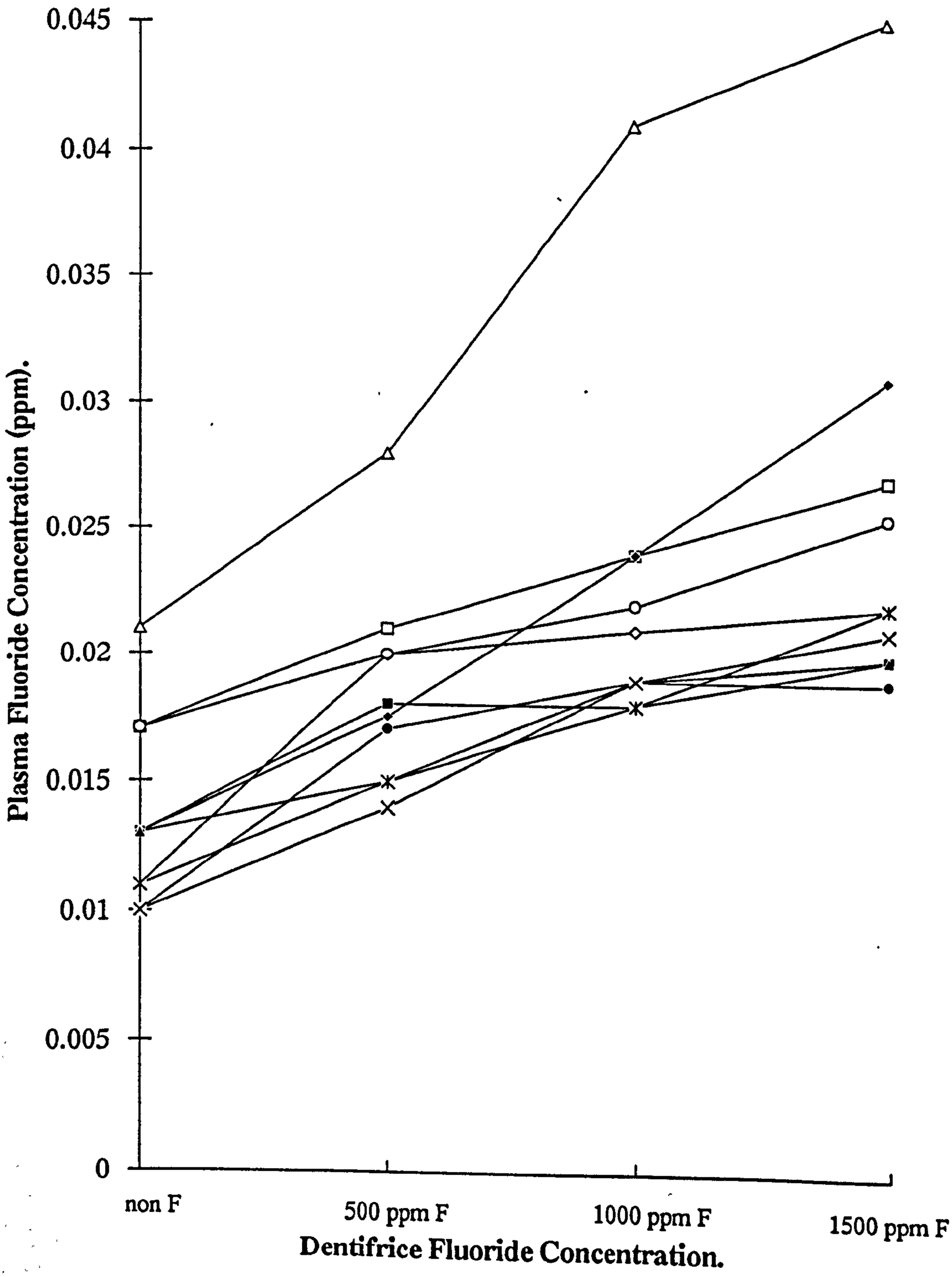


Table 4.16 **Overall mean, (S.E.) and 95% confidence intervals of plasma fluoride versus dentifrice concentration (ppm F).**

Dentifrice	mean	n	S.E.	95% C.I.
non F	0.014	40	0.001	0.012, 0.016
500 ppm F	0.019	40	0.001	0.017, 0.022
1000 ppm F	0.023	40	0.002	0.018, 0.027
1500 ppm F	0.025	40	0.003	0.020, 0.030

Figure 4.8 Mean (95% C.I.) plasma fluoride concentration (ppm) for all 10 subjects (n=40) versus dentifrice fluoride concentration.

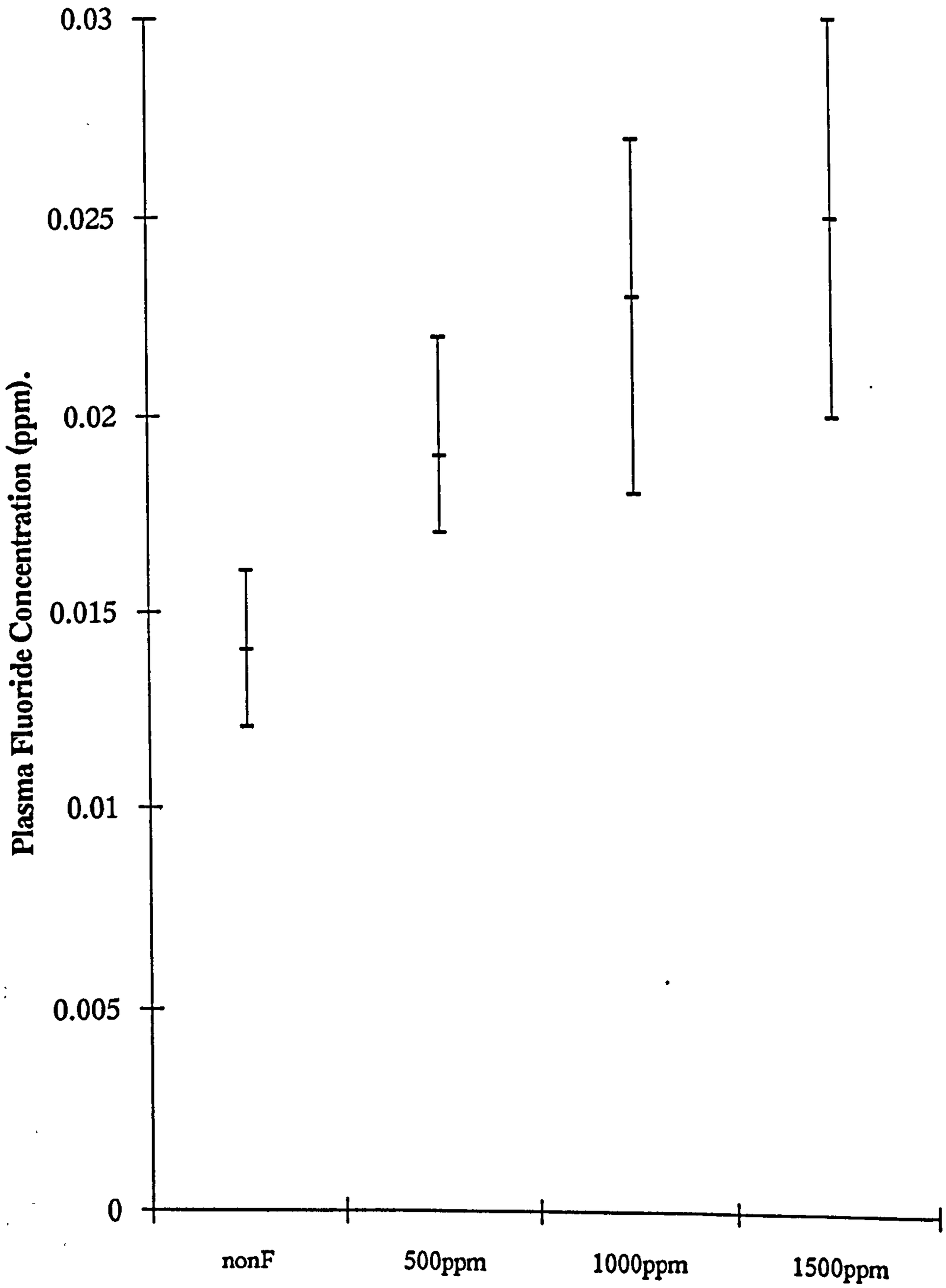


Table 4.17 **Plasma fluoride concentration: Analysis of Variance with dentifrice fluoride concentration and volunteer effects.**

Variable	Degrees of Freedom	Sum of Squares	Mean Square	F Statistic	p value
Dentifrice Fluoride Concentration	3	0.00077	0.00026	32.34	<0.001
Volunteer	9	0.00104	0.00011	14.58	<0.001
Error	27	0.00021	0.00008		
Total	39	0.00202			

4.4.4 Plaque Results.

The mean (n=4) plaque fluoride levels for each of the subgroup of volunteers on each dentifrice is included both in Table 4.18 and Figure 4.9. A general trend of increasing plaque fluoride with increasing dentifrice fluoride was observed.

The overall mean (n=40) for all ten subjects on each dentifrice, the standard error and 95% confidence intervals are tabulated in Table 4.19, and summarised graphically in Figure 4.10.

Finally, two-way ANOVA was performed on the complete plaque data set to identify the influence of dentifrice fluoride concentration on plaque fluoride concentration, in addition to any potential volunteer effects. The results of this ANOVA are presented in Table 4.20, and again demonstrate a highly significant effect of dentifrice fluoride on salivary fluoride ($p < 0.001$), although there appeared to be no significant volunteer effect ($p=0.07$).

4.4.4 Correlation of Salivary, Plaque & Plasma Results.

In an attempt to identify any potential correlation between salivary fluoride levels, plaque fluoride levels and plasma fluoride levels, scatter plots of potential correlations were produced, and are presented in Figures 4.11, 4.12 and 4.13. The corresponding correlation co-efficients are included in Table 4.18.

Table 4.18 **Mean plaque fluoride levels for 10 subjects (n = 4) for each dentifrice concentration (ng/mg wet weight of plaque).**

Volunteer	non-F dentifrice	500 ppm F dentifrice	1000 ppm F dentifrice	1500 ppm F dentifrice
1	1.02	1.08	1.27	1.87
2	1.23	1.33	1.36	1.74
3	1.02	1.60	1.88	2.17
4	1.26	1.31	1.34	1.72
5	1.52	1.67	1.71	1.82
6	1.08	1.58	1.91	2.38
7	1.07	1.05	1.22	2.16
8	0.92	1.25	1.95	2.50
9	1.00	1.03	1.74	1.96
10	0.92	1.31	1.41	1.96

Figure 4.9 Mean plaque fluoride concentration (ng F/mg wet weight) for each of 10 subjects (n=4) versus dentifrice fluoride concentration.

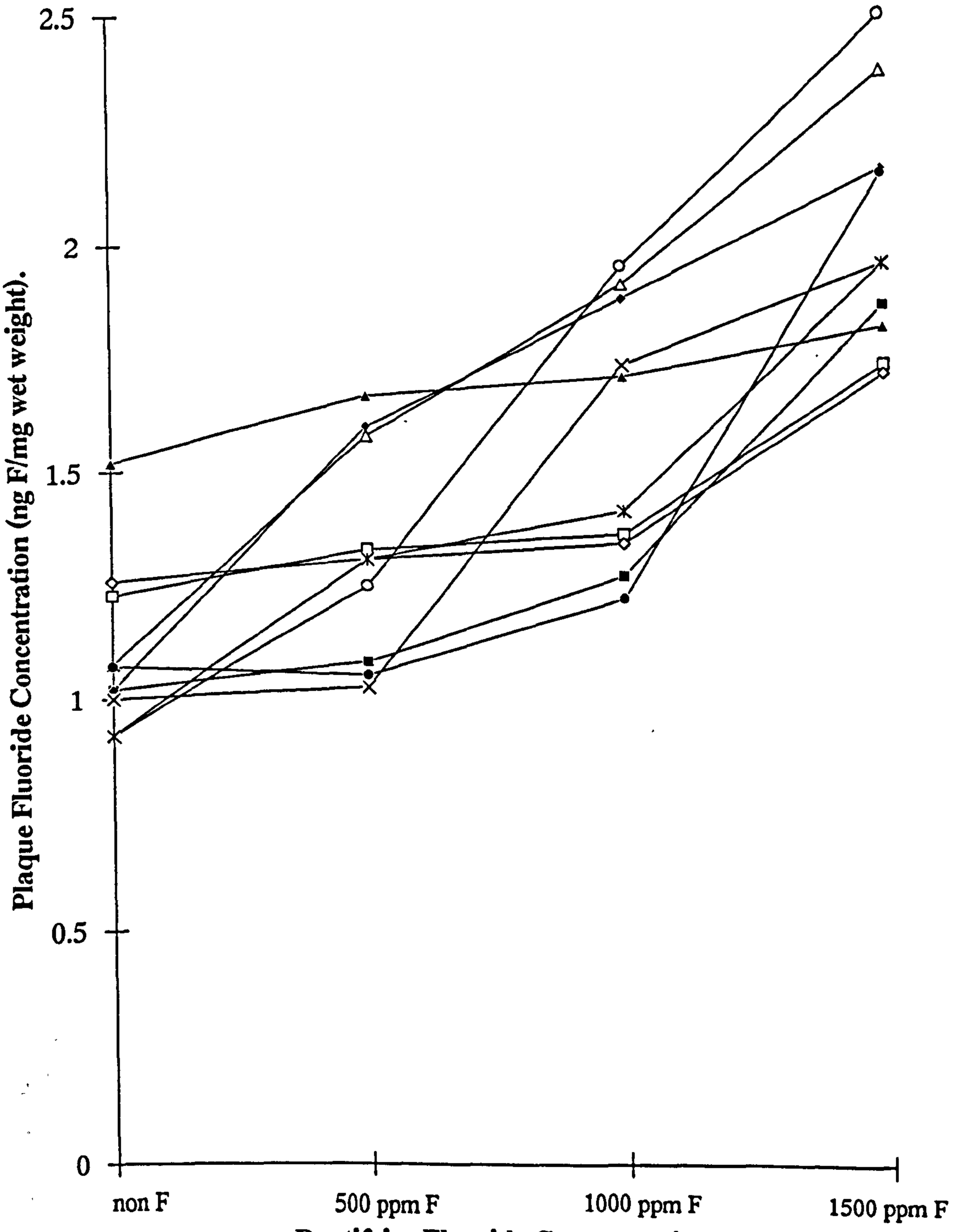


Table 4.19 **Overall mean, (S.E.) and 95% confidence intervals of plaque fluoride versus dentifrice concentration (ng/mg wet weight).**

Dentifrice	mean	n	S.E.	95% C.I.
non F	1.1	40	0.06	0.99, 1.21
500 ppm F	1.32	40	0.07	1.17, 1.47
1000 ppm F	1.58	40	0.09	1.40, 1.76
1500 ppm F	2.03	40	0.08	1.86, 2.20

Figure 4.10 Mean plaque fluoride concentration (ng /mg wet weight) for all 10 subjects (n=40) versus dentifrice fluoride concentration.

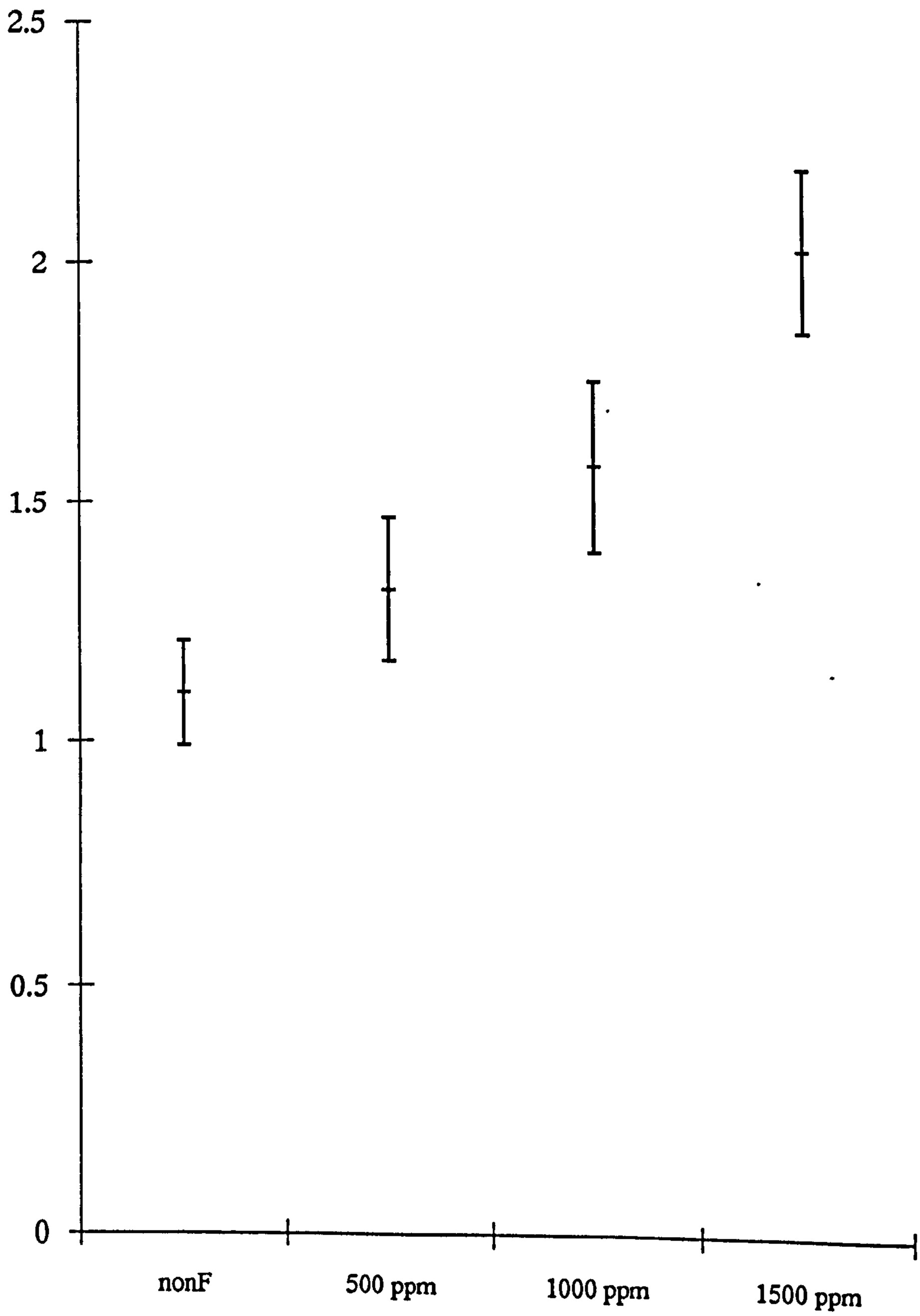


Table 4.20 Plaque fluoride concentration: Analysis of Variance with fluoride concentration and volunteer effects.

Variable	Degrees of Freedom	Sum of Squares	Mean Squares	F Statistic	p value
Dentifrice Fluoride Concentration	3	4.742	1.581	33.14	<0.001
Volunteer	9	0.888	0.099	2.07	0.07
Error	27	1.288	0.0477		
Total	39	6.917			

Figure 4.11 Salivary fluoride (ppm) versus plaque fluoride concentration (ng F/mg wet weight) for 10 subjects.

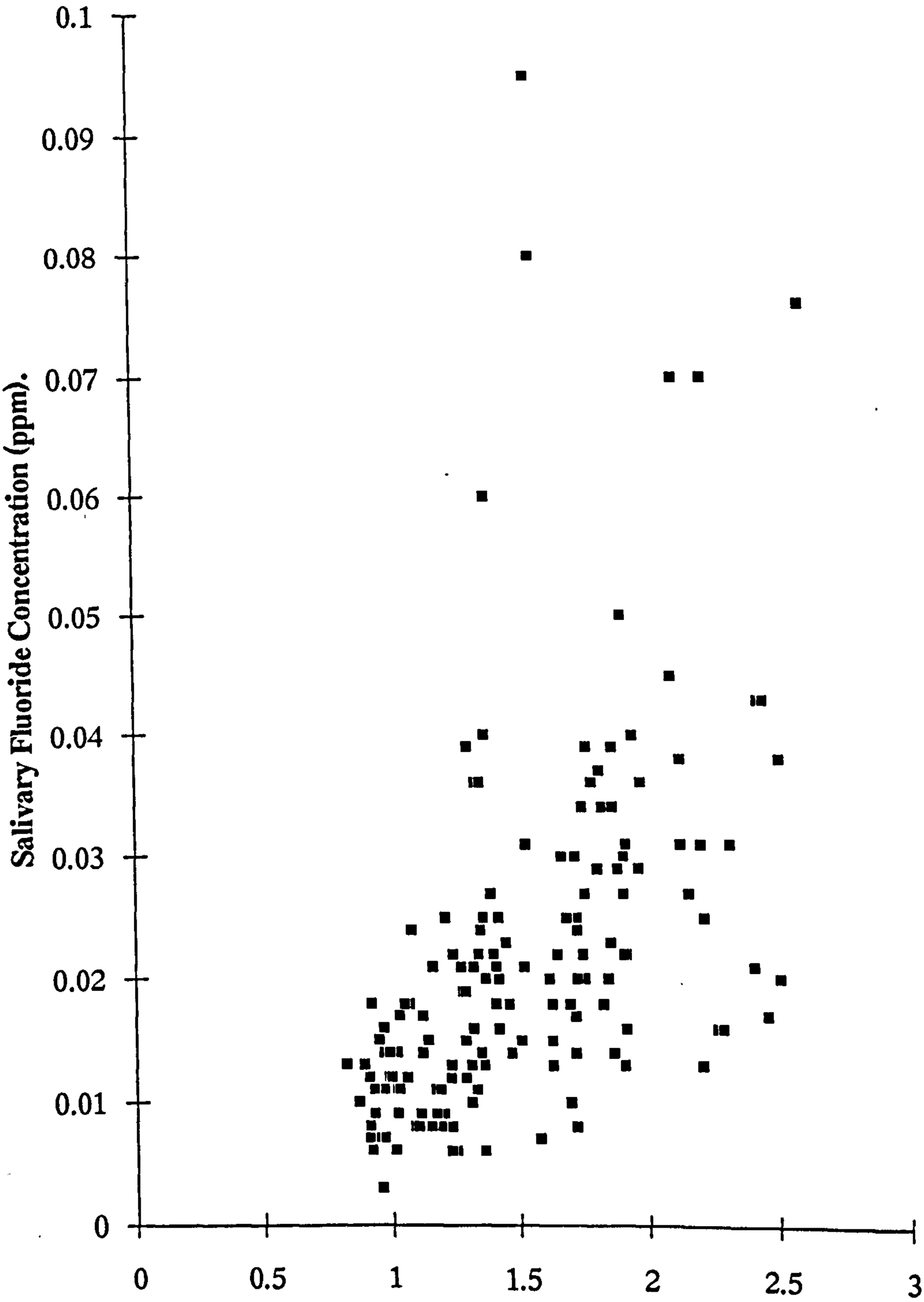


Figure 4.12 Plasma fluoride concentration (ppm) versus salivary fluoride (ppm) for 10 subjects.

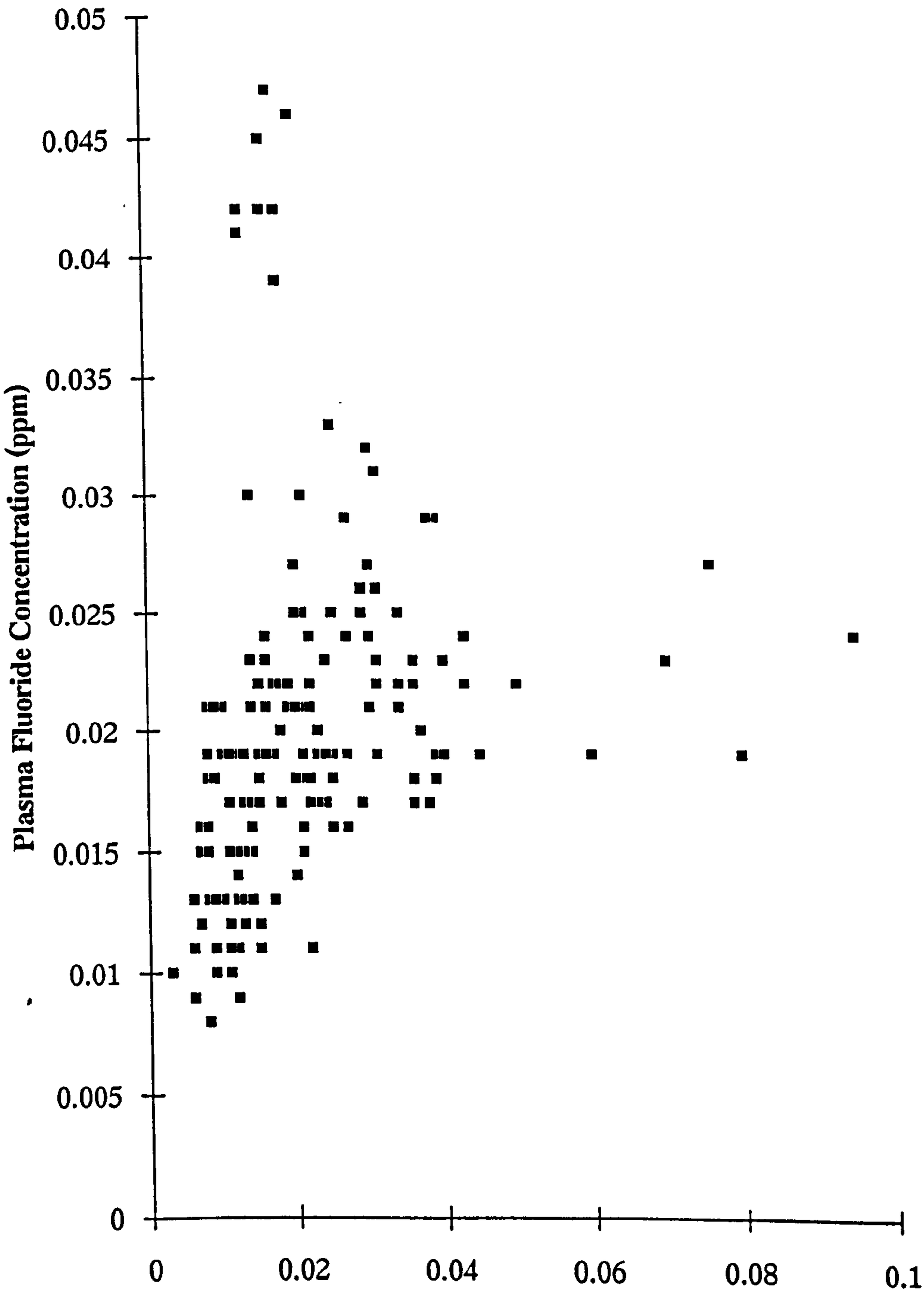


Figure 4.13 Plasma fluoride (ppm) versus plaque fluoride (ng F/mg wet weight) for 10 subjects.

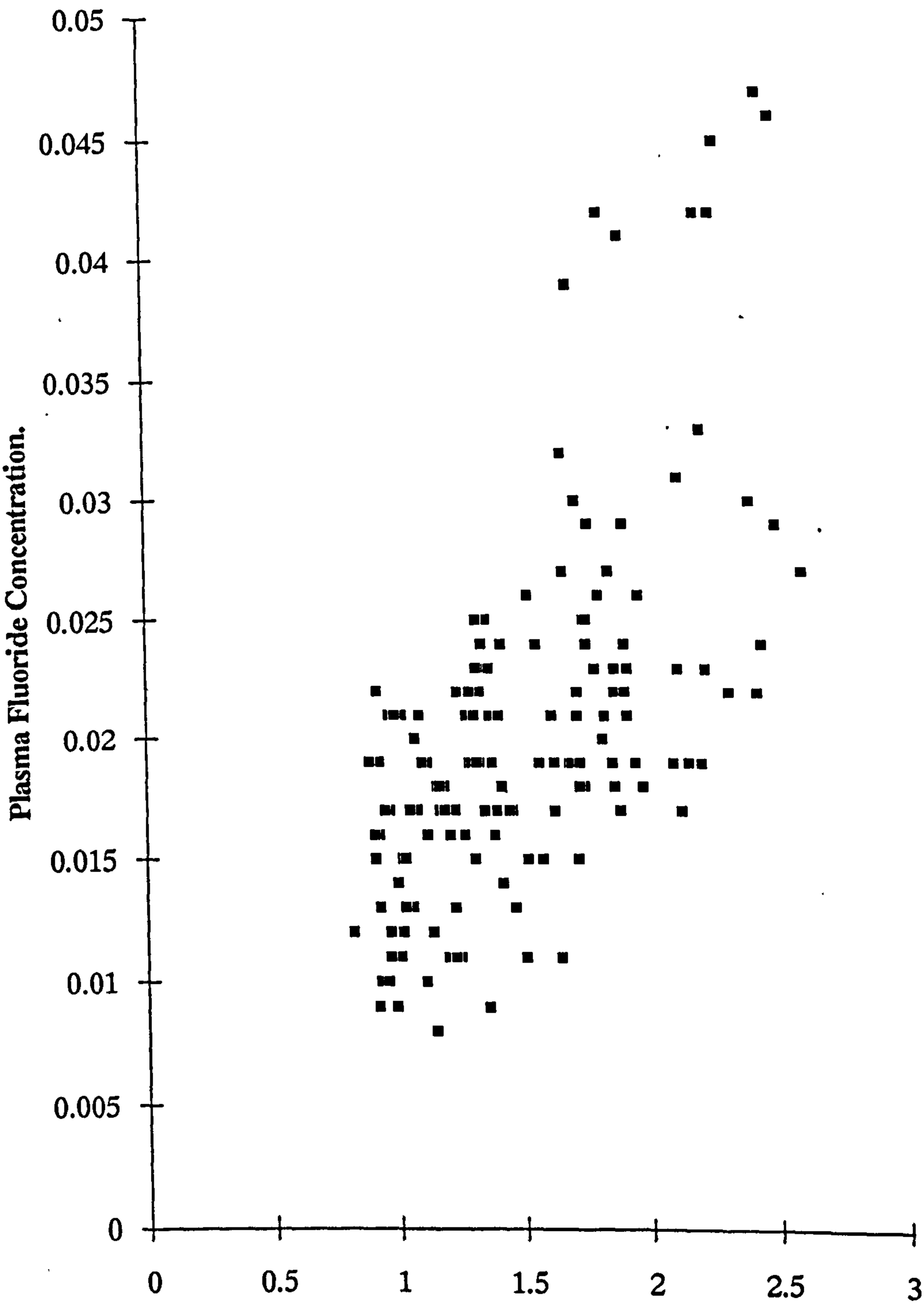


Table 4.21 **Two variable linear correlations between salivary fluoride concentration, plasma fluoride concentration and plaque fluoride concentration. (10 subjects: combined data).**

	Salivary fluoride	Plasma fluoride	Plaque fluoride
Salivary fluoride		0.26	0.52
Plasma fluoride	0.26		0.63
Plaque fluoride	0.52	0.63	

4.5 Discussion.

A number of workers have demonstrated a relationship between the fluoride concentration of a topical fluoride vehicle, and the concentration of fluoride found in mixed saliva and plaque up to 2 hours after the fluoride application (Bruun *et al.*1982; Duckworth, Morgan & Murray,1987; Ekstrand,1987). However, *in vitro* studies have shown that fluoride concentrations as low as 0.04 mg/L have a cariostatic effect (Margolis, Moreno & Murphy,1986) and this study was designed to investigate the influence of the dentifrice fluoride concentration on much lower, basal (i.e. several hours after application) salivary and plaque fluoride concentrations.

4.5.1 Discussion of Salivary Results.

A total of 19 of the original 20 subjects completed the 16 weeks of this study, and the results demonstrate clearly an increase in ambient mixed salivary fluoride concentration as the dentifrice fluoride concentration is raised. Increased variation in salivary fluoride levels was also apparent with increasing dentifrice fluoride content (see Fig. 4.4). For example, the mean salivary fluoride concentrations on the non-F dentifrice ranged from 0.008 - 0.02 ppm, whilst on the 1500 ppm dentifrice the range was from 0.017 - 0.073 ppm. Occasionally, a particularly high reading was obtained e.g. Volunteer 2, (500 ppm dentifrice), reading 10 = 0.06 ppm, and Volunteer 10, (1500 ppm dentifrice), reading 2 = 0.17 ppm (see Appendix 4.1 for raw data and Appendix 4.2 for individual salivary profiles). These 'outlying' results are most readily explained by occasional protocol violations by these individual volunteers, for example, use of the dentifrice closer to sample collection time than prescribed. This problem of volunteer compliance with protocols is common to virtually all aspects of clinical research, particularly during prolonged studies with minimal supervision.

With respect to Table 4.6 and Figure 4.5, these indicate clearly a directional relationship between increasing salivary fluoride concentration and increasing dentifrice fluoride

concentration for the group as a whole, and this is confirmed by the subsequent statistical analysis (Table 4.13).

The initial attempts to establish the extent of a "carry-over" effect (Tables 4.7, 4.8 & 4.9) produced interesting results on the influence of "duration of dentifrice usage", which were verified subsequently by ANOVA (Tables 4.11.1, 4.11.2, 4.11.3). It had been anticipated that with each change in dentifrice fluoride concentration there would be an initial variable period of 5-10 days, followed by a period of relative stability prior to the introduction of the next dentifrice. In fact, the pattern for the non-F dentifrice was a gradual decline in salivary fluoride to detection limit levels within 5-7 days, followed by a gradual recovery to around the pre-experimental level. This phenomenon resulted in the initial two weeks on non-F dentifrice producing significantly different results from the second two weeks.

There may be both a systemic and a local rationale for these findings. The fluoride content of mixed saliva is derived from two sources: firstly, fluoride recirculating from the central compartment, plasma (see Section 1.3.8), via duct saliva, which has a slightly lower fluoride concentration than plasma, and from gingival crevicular fluid, which has approximately 10% more fluoride than plasma (Whitford, Allman & Shahed,1987; Whitford,1989) and secondly, from local, intra-oral sources such as fluoride deposits from dentifrices and mouthrinses, plaque fluoride and enamel. During the initial 3-5 days of this study, when fluoride was virtually withdrawn, all these sources of salivary fluoride would be progressively depleted. However, the skeletal reservoirs of fluoride are extensive, and it has been demonstrated (Neuman & Newman,1958) that the fluoride in the ion-rich aqueous shells of bone crystals is available to the extra-cellular fluids. The fluoride in this pool is rapidly exchangeable, such that it can undergo net migration in either direction, depending on the relative concentrations of the extra-cellular fluid and the hydration shells (Whitford,1989). It seems plausible, therefore, that a shift in concentration gradient initially producing lower plasma fluoride levels, would result in increased release of fluoride from hard tissue stores, such that both

plasma, and indirectly, salivary fluoride levels gradually increase over 2-3 weeks towards the pre-experimental level.

Furthermore, on the re-introduction of fluoride, in the form of the 500 ppm F dentifrice, there is another period of fluctuation. There is an initial "overshoot", where saliva and plasma levels increase rapidly (see Section 4.5.2), to be followed by a gradual stabilisation after 5-7 days. This appears to be the same phenomenon, only in reverse. Again, the results for salivary fluoride over the first 2 weeks were significantly different from the second 2 weeks. In fact, on the introduction of each new dentifrice, there appeared to be a slight "overshoot", although this was not sufficient for the 1000 ppm and 1500 ppm dentifrices to make a statistically significant difference between the early and later phases.

Finally, ANOVA on all the salivary fluoride results (Table 4.13), demonstrates clearly the significant effect of dentifrice fluoride concentration on the basal (i.e. > 3 hours post fluoride application) concentration in saliva ($p<0.001$). If the hypothesis that it is the low, long-term levels of fluoride in the oral cavity which are most important from a caries-preventive perspective then these findings have important implications for the new and largely untested lower level fluoride dentifrices which have been introduced on to the market (Stephen,1994). In Chapter 5 of this thesis, the potential significance of these low salivary levels is addressed further.

4.5.2 Discussion of Plasma Results.

The subgroup of ten volunteers who provided weekly plasma and plaque samples in addition to the x3/week saliva samples, were selected purely on a basis of compliance. The remaining ten subjects were reluctant either to have plaque collected on a weekly basis, or to provide a fingertip blood sample, and in the interests of maintaining as large a group as possible, continued in the study providing the saliva samples only.

The mean plasma fluoride values for each of the ten subjects, on each dentifrice, are presented in Table 4.15. Review of these data reveals that all of the ten volunteers who provided weekly plasma samples showed a progressive increase in fluoride concentration as the dentifrice fluoride concentration increased. Of the ten subjects, nine fell within a similar range, whilst one individual, (Subject 6), had consistently higher levels than the remainder of the group and contributed significantly to the variability within the group.

In Table 4.16 and Figure 4.8, the overall mean for the ten subjects together, on each dentifrice is presented, and this shows a similar pattern to that for saliva i.e. a trend towards a general increase, with an increase in variability in the higher fluoride concentrations. The ANOVA shows highly significant results for both the dentifrice fluoride concentration and volunteer effects ($p<0.001$).

As discussed in Section 1.3.8, the numerical value for the fasting plasma concentration of healthy adults, whose main source of fluoride is the diet, has been defined as roughly equal to that in the drinking water (Guy, Taves & Brey,1976). Therefore, for an adult population living in the West of Scotland where the water fluoridation level is around 0.03 ppm, the anticipated plasma level should be around the same i.e. 0.03 ppm or 1.6 μM/L. Variations from these expected values can be attributed to differences in fluoride intake from other sources, differences in individual rates of renal clearance and differences in rates of exchange between the central plasma compartment and the hard tissue skeleton (Whitford,1994).

In this study, the mean plasma level on the non-F dentifrice was 0.014 (S.E. 0.001), less than the fluoride concentration in the local water supply. It was not until fluoridated dentifrices were re-introduced, at the 1000 and 1500 ppm F levels, that the plasma concentration came close to the local water fluoride level. This could be explained in two ways: either the study population consumed virtually no water during the course of the experiment, including tea, coffee and soft drinks etc. formulated from the local water supply, or the hypothesis given

above (Guy, Taves & Brey,1976), that plasma levels reflect water levels, was based a population which were also utilising fluoridated dentifrices and, in fact, their plasma levels were a reflection of the water supply plus ingestion from topical agents. This latter explanation seems the more plausible.

The potential for systemic absorption of fluoride from topical vehicles is widely discussed in the literature (Hellstrom,1960; Ericsson & Forsman,1969; Hargreaves *et al,*1972; Parkins,1972; Barnhart *et al,*1974; Baxter,1980; Dowell,1981; Wei & Kanellis,1983; Bell *et al,*1985; Bruun & Thylstrup,1988; Simard *et al,*1989; Drummond *et al,*1990; Naccahe *et al,*1990; Levy & Zarei-M,1991; Horowitz,1992; Pang & Vann,1992), and the results generally indicate that an average of 25% (range 10%-100% for individuals) of the fluoride introduced into the mouth with a toothpaste or rinse is ingested. Furthermore, this percentage is higher for young children who do not have good control of the swallowing reflex. For individuals like those in this study population, whose drinking water has a low fluoride concentration, intake from topical fluoride products could exceed intake from the diet, regardless of whether swallowing occurs or not (Whitford, Allman & Shahed,1987). As a direct result of prohibiting post-brush rinsing in this study, the clearance of fluoride from the oral cavity would have been more prolonged than the norm. The sustained contact of the dentifrice/saliva slurry with the oral mucosa may have facilitated accumulation there and subsequent absorption of fluoride directly through the oral soft tissues and eventually into the plasma central compartment. The role of the oral soft tissues in fluoride clearance and absorption is explored further in Chapter 6 of this thesis.

In summary, this study demonstrates clearly a role for topical fluoride in maintaining plasma fluoride levels, most probably via swallowing of the dentifrice but perhaps also via soft tissue absorption.

4.5.3 Discussion of Plaque Results.

The mean fluoride content of the plaque samples for each volunteer on each dentifrice are presented in Table 4.18. These ranged from 0.92 - 1.52 ng/mg wet weight on the non-F dentifrice, to 1.72 - 2.50 ng/mg wet weight on the 1500 ppm dentifrice, and the diagrammatic representation of these data (Figure 4.9) demonstrates the upwards trend of plaque fluoride with dentifrice fluoride, although the increasing variability identified in the salivary and plasma data is less evident.

The overall mean, for the subset of ten volunteers as a whole, on each dentifrice is given in Table 4.19 and Figure 4.10. The mean values were 1.1, 1.32, 1.58 and 2.03 ng/mg wet weight of plaque, for the non-F, 500 ppm F, 1000 ppm F and 1500 ppm F dentifrices respectively, with a slight increase in variability with increase in dentifrice fluoride.

The ANOVA confirmed a highly significant effect of dentifrice fluoride on plaque fluoride concentration but, in contrast to the previous analyses, there appeared to be no significant volunteer effect (p=0.07).

Plaque fluoride is potentially the most complex of the three parameters measured in this study. As discussed in Section 4.1.1, the literature reports total plaque fluoride concentration ranges from 5-50 ppm (equivalent to ng/mg wet weight), of which approximately 15-75% may be detected by an ion-specific electrode in conjunction with suitable buffers, such as were used in this study. That is to say, the range of ionisable, or 'loosely-bound' plaque fluoride quoted in the literature is 0.75-37.5 ppm (i.e. 15% of 5 ppm - 75% of 50 ppm). The range of plaque fluoride found here is in general agreement with that reported in the literature, and in particular correlates with a recent study undertaken using very similar dentifrice formulations (Duckworth *et al.*1994).

Furthermore, in a multi-centre study undertaken in two sites in the U.K. and one site in the

Netherlands, Edgar, Ingram & Morgan (1992) reported 'ambient' salivary and plaque fluoride levels from subjects using placebo and 1500 ppm MFP dentifrices, for a period of four weeks each. The three study sites were supplied with water containing fluoride at three different levels, namely 0.026 ppm (Port Sunlight, U.K.), 0.16 ppm (Amsterdam, the Netherlands) and 1.0 ppm (Birmingham, U.K.). The results showed a mean salivary fluoride level (on the placebo paste) of 0.009 for Port Sunlight i.e. less than the water fluoridation level, 0.013 for Amsterdam and 0.0175 for fluoridated Birmingham. The plaque fluoride levels while on the placebo paste were 1.41, 4.34 and 6.96 ng/mg wet weight of plaque respectively. On the introduction of the 1500 ppm MFP paste, the mean saliva values were 0.016, 0.018 and 0.026 ppm, and the plaque fluoride values had risen to 1.92, 5.28 and 8.57 ng/mg wet weight.

The data from the Port Sunlight group, with a water fluoridation level similar to the West of Scotland (0.03 v 0.023 ppm), provided an interesting comparison to that in the current study. The mean saliva and plaque values on the placebo dentifrice are very similar, 0.011 and 0.009 ppm for saliva, and 1.1 and 1.4 ng/mg wet weight for plaque. A comparison of the 1500 ppm dentifrice data is more difficult, as that in the current study was NaF-based, whilst the Edgar, Ingram & Morgan study used MFP. However, the corresponding saliva fluoride levels were 0.041 ppm for the NaF dentifrice and 0.016 ppm for the MFP dentifrice, with plaque fluoride levels at 2.03 (NaF) and 1.92 (MFP). These data are consistent, given the widely accepted greater bio-availablity of fluoride from NaF dentifrices (Duckworth *et al.*1994; Stookey *et al.*1985; Reintsema, Schultof & Arends,1985; Shellis & Duckworth,1994). The authors concluded that, in the absence of a topical source of fluoride, most of whole salivary fluoride is glandular in origin, but the additional fluoride in whole saliva from a topical application is mostly that retained locally, and not recycled via the salivary glands. They speculated further that this localised retention was due to fluoride adsorbed onto the tooth surface, but were confounded by their own observations of similar

results in edentulous individuals. These observations also suggest a minor contribution from gingival crevicular fluid to salivary fluoride levels. The possibility of soft tissue fluoride adsorption was not explored.

These results lend further support to the suggestion that plaque fluoride is largely dependent on the fluoride intake from topical agents, and from the diet where that contains appreciable quantities (Jenkins & Edgar,1977; Ingram & Morgan,1987).

In conclusion, varying the fluoride content of dentifrices in the range 0 - 1500 ppm F, significantly influences the ambient level of fluoride in whole saliva, in plaque and in plasma and may, therefore, have an important impact on the caries susceptibility of enamel. These findings are particularly pertinent, at a time when non-clinically-proven dentifrices, with lower levels of fluoride, are being introduced.

Chapter 5. *In vitro* pH-cycling Studies: The Influence of Fluoride Concentrations in De- and Remineralising Solutions.

5.1 Introduction & Aims.

A wide range of techniques has been developed to elucidate the mechanisms of enamel remineralisation and demineralisation, and to study how factors such as fluoride concentration influence these mineral changes. These include *in vitro*, *in situ* and *in vivo* models, which all have their proponents, and particular applications. Whilst *in vivo* studies most closely relate to nature, they are often fraught with problems in terms of ethical approval and the provision of adequate controls (von der Fehr, Löe & Theilade,1970; Edgar *et al.*1978). *In situ* and *in vitro* studies, however, pose no major ethical problems, are relatively easy to control and are, therefore, considerably more versatile. For these reasons, they have largely superseded *in vivo* techniques as the preferred methodologies for the determination of the mechanism of action and efficacy of fluoride products.

In this chapter the advantages and disadvantages of these techniques are described and the methods of measuring mineral changes reviewed. In addition, details of the pH-cycling experiment investigating the influence of low concentrations of fluoride in the de- and remineralising solutions will be provided, specifically those low levels of fluoride identified in saliva in the preceding two chapters.

5.2 Remineralisation/Demineralisation Studies.

5.2.1 *In vivo/in situ* Studies.

A number of *in vivo* studies (von der Fehr,1967; Backer-Dirks,1966) have demonstrated

comparison of test and control treatments in the same oral environment, although caution should be exercised when interpreting data from contralateral sites (Damato,1990).

Several variations on this original model have been developed subsequently, which allow plaque accumulation on the enamel specimens, in some instances facilitated by the use of Dacron gauze or steel mesh (Wefel *et al.*1987). Furthermore, a number of authors (Featherstone *et al.*1982; Corpron *et al.*1986; Creanor *et al.*1986a; Hellwig, Klimek & Wagner,1987; Damato & Stephen,1994) have described a technique whereby fully dentate individuals wear a small removable appliance on to which specimens are mounted in a lingual recess, with access openings to allow the diffusion of plaque and salivary constituents to the experimental site.

Most *in situ* models were developed to determine the uptake and incorporation of fluoride into enamel (Koulourides *et al.*1974; Mobley,1982; Mellberg & Chomicki,1983; Stookey *et al.*1985; Zimmerman *et al.*1985; Corpron *et al.*1986; ten Cate & Rempt,1986; Clarke *et al.*1988) when this was considered to be the primary mechanism of fluoride action. Several investigations, including many of the above, additionally demonstrated changes in the mineral content of the enamel specimens. More recently, *in situ* models have been used to compare different fluoride products, and to study the dose-relationship between fluoride concentration and *in situ* remineralisation of enamel specimens (de Kloet *et al.*1986; Goorhuis & Purdell-Lewis,1986; ten Cate & Rempt,1986; Creanor *et al.*1987; Schafer,1989; Damato,1990).

The choice of hard tissue substrate is important in determining the overall sensitivity of an *in situ* technique, and should also be a factor in the interpretation of any results. The enamel specimen may be prepared in a variety of ways, for example natural or artificial lesions, bulk enamel slabs or thin single sections, abraded or unabraded enamel surfaces. Each of these options have their advocates.

One of the more popular options is to use blocks of sound enamel cut from extracted premolar teeth (ten Cate & Duijsters,1982; Featherstone,1983; Zimmerman *et al.*1985; Arends & Dijkman,1988). Two or more blocks can be cut from the same tooth and assigned to the various treatment groups (Featherstone *et al.*1983; Hicks, Flaitz & Silverstone,1986; Schäfer,1989), but this still has the limitation of requiring a separate area of enamel as a control. This may not be sufficient to account for all the variations in lesion formation or remineralisation, since there can be considerable variation in demineralisation within different areas of the same tooth (Strang *et al.*1986; Larsen,1990). Variations in the mineral content between cervical and coronal enamel have also been identified (Theuns, Arends & Groeneveld,1980; Theuns *et al.*1983) and have been related to the degree of demineralisation found in these areas (Groeneveld & Arends,1975; Creanor, Strang & Stephen,1989). It is well established that enamel is non-homogenous, and marked differences in mineral and trace element content are present even in the same tooth (Robinson, Weatherell & Hallsworth,1971) which may explain some of the variability encountered in the studies above. Similarly, variations amongst teeth of different ages have also been reported (Woltgens *et al.*1981; Woltgens *et al.*1983; Kidd *et al.*1984).

The development of the single-section technique (Featherstone & Silverstone,1982; Anderson & Elliot,1987; Mellberg, Castrovince & Rotsides,1985; Wefel & Harless,1985; Creanor *et al.*1986b; Strang *et al.*1987; Wefel *et al.*1987) has largely overcome the problem of enamel variability. With the use of thin sections of enamel, the same area can be studied longitudinally, and each section/lesion therefore acts as its own control. The main disadvantages of the single-section technique are logistical ones, in that it is very labour-intensive and there is a risk of damaging the enamel sections during their removal from the appliance, radiographing and revarnishing at the end of each treatment phase. However, the enhanced sensitivity of the technique which results from the elimination of the problem of enamel non-homogeneity, renders it the chosen methodology for the *in vitro* section of this

project.

In future, more sensitive analytical techniques may render *in vivo* protocols a more realistic proposition e.g. refinements of X-ray image analysis, transillumination, scanning optical monitoring, electrical resistance and fluorescence techniques (Arends & ten Bosch,1986; de Josselin de Jong *et al.*1988), but until these are more readily available *in situ* and *in vitro* techniques offer the best alternatives.

A review of *in situ* remineralisation studies (Arends & ten Bosch,1986), indicated that the complex oral environment has a great deal of influence on the de-/remineralisation equilibrium, and that results often vary from those which *in vitro* studies would predict. Well-defined thermodynamic conditions can be obtained in the *in vitro* studies, and the kinetics of both de- and remineralisation can be studied. The presence of saliva and plaque in the *in situ* studies not only complicates the inorganic ion profiles, but provides organic components which may complex ions, absorb to surfaces, act as inhibitors of both de- and remineralisation, and modify diffusion in and out of enamel, root dentine and plaque fluid. The *in situ* environment does not lend itself to well-defined conditions, where variables may be manipulated one at a time, to show their influence on the caries process. This makes mechanistic studies much more difficult in the oral environment than in the well-controlled laboratory situation.

5.2.2 *In vitro* Studies.

Initially, *in vitro* studies concentrated on the effect of fluoride on surface softened enamel (Pigman, Cueto & Baugh,1964; Feagin *et al.*1971), and such variables as pH, ionic strength and calcium, phosphate and fluoride concentration were studied for their influence on early remineralisation. Subsequently, however, remineralisation studies have concentrated on subsurface lesions, as a more accurate representation of natural caries (ten Cate & Arends,1977; Groeneveld, Theuns & Kalter,1978; Clarkson, Wefel & Feagin,1986).

The technique of pH cycling has been developed in an attempt to simulate the fluctuating conditions of the enamel environment. *In vivo,* following consumption of fermentable carbohydrate, the pH in plaque shows a sharp decline, followed by a slow increase to physiological levels (Stephan,1944). This results in a series of approximately 30 minute depressions in pH, each followed by a "resting" period and results in a dissolution and reprecipitation of the tooth mineral. During this process, calcium phosphates such as hydroxyapatite (HAP), which are stable at neutral pH, may be converted to other minerals e.g. brushite, which are more stable at lower pHs (ten Cate,1990). Using a pH cycling model, ten Cate & Duijsters (1982) demonstrated that although fluoride initially enhanced remineralisation, it eventually halted both de- and remineralisation, providing a possible explanation for the observation of lesion arrestment *in vivo.*

In *in vitro* pH cycling models, enamel specimens are usually exposed to one demineralisation cycle and one remineralisation cycle per 24 hours, for a period of four or more weeks. By altering the pH of the remineralising medium, the efficiency of fluoride incorporation into enamel can be increased (Brudevold *et al.*1963; Duff,1976). This observation was the scientific basis for the clinical introduction of fluoride-containing acidulated phosphate (APF 1.23% F) gels. A significant amount of information on the mechanism of enamel de- and remineralisation has been obtained using *in vitro* techniques, including rates of crystal formation, ion diffusion and surface layer phenomenon (ten Cate & Duijsters,1982; Featherstone *et al.*1986; Gerrard & Winter,1986; ten Cate & Simmons,1986; White,1987; ten Cate *et al.*1988; Damato, Strang & Stephen,1988).

5.3 Assessing Changes in Mineral Content.

A number of different techniques are employed currently to measure the mineral content of dental enamel. Those most commonly quoted are: (i) microradiography (Angmar, Carlström & Glas,1963; Groeneveld,1974; Arends & Gelhard,1983; Gelhard & Arends,1984a; de

Josselin de Jong & ten Bosch,1985; Strang *et al.*1987); (ii) polarising microscopy (Darling,1956; Silverstone,1968); (iii) microhardness (Arends, Schultof & Jongebloed,1980; Featherstone *et al.*1983), and (iv) chemical measurements (ten Cate & Duijsters,1982; Borsboom, van der Mei & Arends,1985).

5.3.1 Transverse (Contact) Microradiography/Microdensitometry.

Microradiography and microdensitometry techniques are founded on the principal that the degree of absorption of X-rays by an enamel section will depend on the mineral content of that tissue. Angmar, Carlstrom & Glas (1963) developed a theory for calculating enamel mineral content from microdensitometric measurements of microradiographic images of enamel, and Bergman & Lind (1966) extended the technique to assess the mineral content of demineralised enamel.

In general, quantitative microradiographic analysis of the carious lesion depends on the fact that demineralised enamel will absorb fewer X-rays than sound enamel, and the optical density (greyness) of a radiographic image of the enamel is dependent on the mineral content. Subsequent microdensitometric analysis enables the grey levels to be quantified into mineral content. According to Angmar *et al.,* (1963) the grey level for any point in a lesion results from the X-ray absorption by two major components, namely "t_m", the inorganic component thickness, and "t_o", the organic component thickness. Hence for a particular level of absorption of X-rays i.e. the grey value of a point in a lesion, the absorption in enamel can be equated against the absorption in an aluminium step-wedge. In deriving the equation, Angmar *et al.* (Angmar, Carlström & Glas,1963) made the assumptions that (i) mineral salts have a density of 3.15 and (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.

Certain conditions must be fulfilled to ensure the accuracy of this technique. Firstly, monochromatic radiation must be used: this is achieved by using a nickel filter which absorbs

all unwanted X-rays from the polychromatic source. Secondly, the enamel sections must be plano-parallel to enable a single value of section thickness to be used in calculations and thirdly, since the intensity of the incident X-ray beam is unknown, a reference system must be incorporated for calibration purposes. In this study, the reference medium was an aluminium step wedge which was radiographed along with the enamel sections. Finally, the X-ray beam must be homogenous and of sufficient width. The intensity of the X-ray beam used in this work varied by 13% in the vertical axis, and by 1% in the horizontal axis.

Microradiography/microdensitometry has been described as the ideal technique for the assessment of hard tissue mineral content. It is non-destructive and provides a direct measurement of mineral content, in contrast to a variety of alternative methodologies, details of which are summarised below. Transverse microradiography of single sections, however, assumes the section is homogeneous in thickness. White *et al.* (1992) have demonstrated that achieving such homogeneity in single sections of 100-200 μm can be problematic. Further difficulties are encountered when remineralising protocols call for inclusion of ions with a very high absorption co-efficient for X-rays, for example tin ions, which may lead to a misinterpretation of results (Arends & ten Bosch,1992).

5.3.2 Longitudinal Microradiography.

This technique involves samples of teeth up to 0.5 mm thick, which are cut parallel to the anatomical surface and exposed, along with an aluminium step wedge, to Cu-K_α radiation generated by 20 keV electrons. The use of a microdensitometer facilitates the determination of the mineral content of a lesion per unit area. Mineral loss is expressed in terms of % vol. x μm or kg/m^2. The method is thought to be subject to errors of up to 20% (ten Bosch & Angmar-Månsson,1991). This method can now be applied to natural tooth surfaces, although variations in thickness of specimen should not exceed 200 μm (de Josselin de Jong, van der Linden & ten Bosch,1987). The exposure of the anatomical surface of the lesion to

the X-ray source, rather than through the cross-section, means it is not possible to derive information on the variation of mineral content at various depths throughout the lesion.

5.3.3 Wavelength Independent Microradiography.

Polychromatic, high energy X-rays (≥ 60 kV) are used for non-destructive mineral determination of whole teeth. A reference step wedge is also used for this technique to enable calculation of mineral content. This method can measure, with great accuracy, the amount of mineral per unit area in enamel or dentine samples with a thickness of between 0.3 mm and 6.0 mm, and with either natural or curved surfaces. Accuracy is reduced when mineral determination of whole teeth is attempted, rather than blocks. Mineral loss is expressed in units of % vol. x μm or kg/m^2 (Arends & ten Bosch,1992). Once again, this technique has the disadvantage of being unable to determine the change in mineral content in the depth of a lesion.

5.3.4 Polarising Microscopy.

Polarising microscopy is a simple, and when used quantitatively, non-destructive method of studying mineral distribution in carious enamel. The technique utilises the birefringence of enamel mineral to demonstrate changes in enamel porosity. Enamel is said to have a negative intrinsic birefringence (value 1.62) due to its orientated crystal component, and a positive form birefringence due to the presence of small, orientated pores which can be altered by changing the imbibing medium.

When sections of enamel are to be examined in polarised light, they are first placed in an imbibition medium e.g. water, quinoline or naphthalene. The magnitude of form birefringence is dependent on the refractive index of the solid of the medium filling the pores and on the volume fraction occupied by the solid. Thus, when carious enamel is examined after imbibition in water, the form birefringence will be produced if the spaces created in the

tissues are large enough to admit water. The observed birefringence, which is the sum of the intrinsic birefringence and the form birefringence, can be measured using a suitable optical compensator in conjunction with a polarising microscope.

The histological pattern of carious enamel examined using this technique has been described in some detail (Silverstone,1968; Kidd,1983). Here, four separate areas were distinguished, namely the surface zone, the translucent zone, the dark zone 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 mineral deposition within. The micropore system of the dark zone has been described as a "molecular sieve" (Darling *et al.*1961; Poole, Newman & Dibdin,1981) and is considered the resting point for mineral which has been removed from the deeper layers of the lesion. As mineral is progressively deposited in the dark zone and in the surface zone, these areas are considered to become broader and more well-defined (Kidd,1983).

This technique has been applied frequently (Silverstone,1968; Kidd,1983) as it gives much ultrastructural detail (see Figure 1.1) although it is considered to be only semi-quantitative. It has been shown (Shellis & Poole,1985) that estimation of pore volume from form birefringence is not entirely reliable, as the pore distribution within the lesion appears to be more random than was assumed originally. As a method for estimating enamel mineral content it is therefore only advisable to use this technique in conjunction with other calibrating methods, such as microradiography.

5.3.5 Light Scattering.

Scattering of light within a lesion makes incipient lesions look whiter than surrounding sound enamel. This phenomenon is due to the mineral in an enamel lesion being surrounded by lesion fluid, rather than by other sound enamel mineral (ten Bosch & Angmar-Månsson,1991). Methods involving optical fibre technology enable such light scattering to be quantified and correlated with mineral loss (ten Bosch, van der Mei & Borsboom,1984).

This technique is now being applied to *in vivo* caries assessment.

5.3.6 Microhardness.

The microhardness technique was initially developed to assess the hardness of homogenous materials and was introduced only later for experimental caries studies (Caldwell *et al.*1958; Newbrun, Timberlake & Pigman,1959) under the assumption that the measured hardness is related to the degree of porosity of the superficial enamel. The technique involves the measurement of the depth of penetration of a Knoop diamond under a fixed load into enamel. On a non-elastic material the diamond indents the surface, and the maximum length of this indent defines the penetration depth. The indenter must descend in a perpendicular direction onto a horizontal plane and the test surface must remain intact as seen by microscopic examination (x 200 - 500 magnification). This is usually carried out on normal bulk enamel (Koulourides *et al.*1974; Gelhard, ten Cate & Arends,1979; Arends, Schultof & Jongebloed,1979; Arends, Schultof & Jongebloed,1980), or on polished cut surfaces (ten Cate, Shariati & Featherstone,1985). Featherstone and co-workers (1983) have shown that, when using polished, cut surfaces, the square root of the Knoop Hardness Number (KHN) calculated from the indentation length, is linearly proportional to the volume percent mineral determined by microradiography. The disadvantages of this technique include: (i) a requirement for a polished surface (ii) it is time-consuming, (iii) its validity depends on the assumption that the change in mineral density is similar at all points on the test surface, (iv) because the method is destructive, a separate control specimen is always required and (v) there is a poor spatial relationship between mineral change and lesion depth.

5.3.7 Chemical Techniques.

The most frequently quoted chemical techniques for assessing mineral change are :

(i) the monitoring of calcium and phosphate concentrations in solutions into which enamel

slabs have been placed under specific conditions,

(ii) the "enamel biopsy" technique and

(iii) iodine permeability.

Calcium in small samples is most conveniently measured using atomic absorption spectrometry, whilst phosphate is generally measured colorimetrically (Chen *et al.*1956). A good correlation has been found between the calculated rate of demineralisation from microradiography and chemical analysis (ten Cate & Duijsters,1983; Theuns *et al.*1985). However, the rates of demineralisation obtained from chemical analyses are found to have smaller standard deviations than those obtained from microradiography, since local variations in enamel specimens have little influence on the chemical results. The major disadvantage of this technique is that no mineral profile is produced, and therefore the areas within a lesion which are demineralising cannot be identified.

The "enamel biopsy" technique was first described by Brudevold, McCann & Grøn (1968), and has largely been used to determine the fluoride concentration of surface enamel, rather than the calcium and phosphate content. The original methodology was based on mechanical grinding of the surface enamel, and was later modified (Hotz, Mühlemann & Schait,1970; Munksgaard & Bruun,1973; Weatherell, Hallsworth & Robinson,1973) to utilise acid to etch sequential layers from the surface. Microdrilling (Hallsworth, Weatherell & Robinson,1973; Sakkab, Cilley & Haberman,1984) and abrasion techniques (Weatherell *et al.*1977) have also been reported.

All these methods involve the dissolution of samples of hard tissue in acid and their analysis for a range of inorganic ions by atomic absorption spectrophotometry or other colorimetric techniques. They have proved to be very sensitive methods for detecting mineral change, but are time-consuming, destructive and do not provide information regarding change in mineral

content with depth of the lesion.

The iodine permeability (Ip) technique was first published in 1977 by Bakhos, Brudevold & Aasenden, and, strictly speaking, depends on a physical, rather than a chemical change in the test material. When evaluated *in vitro*, Ip values were found to vary considerably when comparing different enamel samples (Bakhos & Brudevold,1982). However, for each sample there was a consistent increase in Ip with increase in acid challenge, and the relationship between Ip and dissolved Ca was found to be linear at all pH levels tested (4.0, 4.5 and 5.0). Brudevold *et al.,* (1982a; 1982b) reported that Ip measurements were related to pore volume of the enamel, and that the Ip test can give sensitive estimations of the initial stages of de- and remineralisation. The Ip test has been validated against surface microhardness (Zero *et al.*1990) and proved to be sufficiently sensitive to detect the very earliest stages of enamel demineralisation, although it was considerably more variable, is regarded as operator-sensitive and does not produce a mineral profile.

In view of the greater accuracy afforded by microradiography, and the ability of this technique to produce a profile of mineral change with lesion depth, this was the technique employed for mineralised tissue assessment in the work reported in this thesis.

5.4 Experimental Protocol.

The aim of this *in vitro* experiment was to determine if the range of mean fluoride concentrations found in mixed saliva *in vivo*, and described in the preceding chapter, could have any influence on the re- and demineralisation of early enamel lesions in a pH-cycling model. The remineralising phase was 21 hours in duration, the demineralising phase was 3 hours, and four groups of sections were cycled through these phases every 24 hours, over a 5 week period. To minimise any potential carry-over from one phase to the next, the sections were washed in distilled, de-ionised water before each change. Fluoride, as NaF, was added to both the demineralising and remineralising solutions of each group as follows:

Group 1 - 0 ppm F; Group 2 - 0.02 ppm F; Group 3 - 0.04 ppm F and Group 4 - 0.06 ppm F. On the 7th day of each week, all the sections were removed from their carriers, cleaned of varnish, radiographed, repainted and remounted on the carriers, to begin another week of the study.*

5.5 Materials & Methods.

5.5.1 Enamel Source.

The tooth sections used in this study were obtained from human premolars extracted for orthodontic purposes in various dental practices in the West of Scotland (water fluoride concentration <0.03 ppm). Some workers use bovine rather than human enamel, as it homogeneous and easily obtained. However, Featherstone & Mellberg (1981) compared the demineralisation of human, ovine and bovine enamel, and found marked differences in the rate of demineralisation. In addition, Poole, Shellis & Tyler (1981) found that the rate of lesion formation was greater in the enamel of non-human primates than in man, and attributed this difference to the lower porosity of human enamel. It was decided, therefore, for the purposes of this thesis human enamel was the substrate of choice.

5.5.2 Tooth Preparation.

Following extraction, the premolars were stored in plastic containers with 0.1% thymol solution. Prior to lesion creation, teeth were washed in soapy water, and cleaned with a pumice/alcohol mixture to remove any pellicle and debris. The pumicing procedure was performed manually and was not intended to remove any significant depth of surface enamel. After thorough rinsing in cold running water the teeth were air-dried. A length of adhesive tape (width 40 μm) was wrapped around each tooth four times, leaving a gap of approximately 300 μm between each strip. Thereafter, two coats of acid-resistant nail varnish (*Revlon*) were applied to the crown of the tooth, which was then left to air-dry. The

(* Damato, Strang & Stephen, 1990.)

adhesive tape was then removed, leaving four exposed windows across the buccal surface. The remainder of each tooth, including the root(s), was then varnished and again left to air-dry. Finally, each tooth was examined using a stereomicroscope with x 10 magnification, to ensure exposed areas were free of any adhesive or varnish, prior to immersion in a demineralising solution.

5.5.3 Preparation of Subsurface Lesions.

The demineralising solution (pH 4.5, pI* 126) was prepared regularly in 2 litre batches. It contained 3.1 mM each of calcium chloride and sodium dihydrogen orthophosphate and 50 mM (2.9 mL) glacial acetic acid. Double-distilled, deionised water was added and the pH adjusted up to 4.5 with 1 M sodium hydroxide. The solution was then transferred to a volumetric flask, and made up to 1 litre with additional double-distilled, deionised water. Batches of five varnished teeth, each with four exposed windows, were placed in 50 ml of the demineralising solution. This solution was changed after 48 hours and the teeth removed from the demineralising solution after a further 72 hours. The teeth were then washed several times with acetone to remove the varnish. The fluoride content of the demineralising solution was measured regularly and found consistently to be below 0.02 ppm. Teeth were judged to be sufficiently demineralised when the air-dried surface appeared to be a dense, opaque white colour whilst retaining its surface shine and integrity (Figure 5.1). Teeth that became cavitated or failed completely to demineralise during this period were discarded.

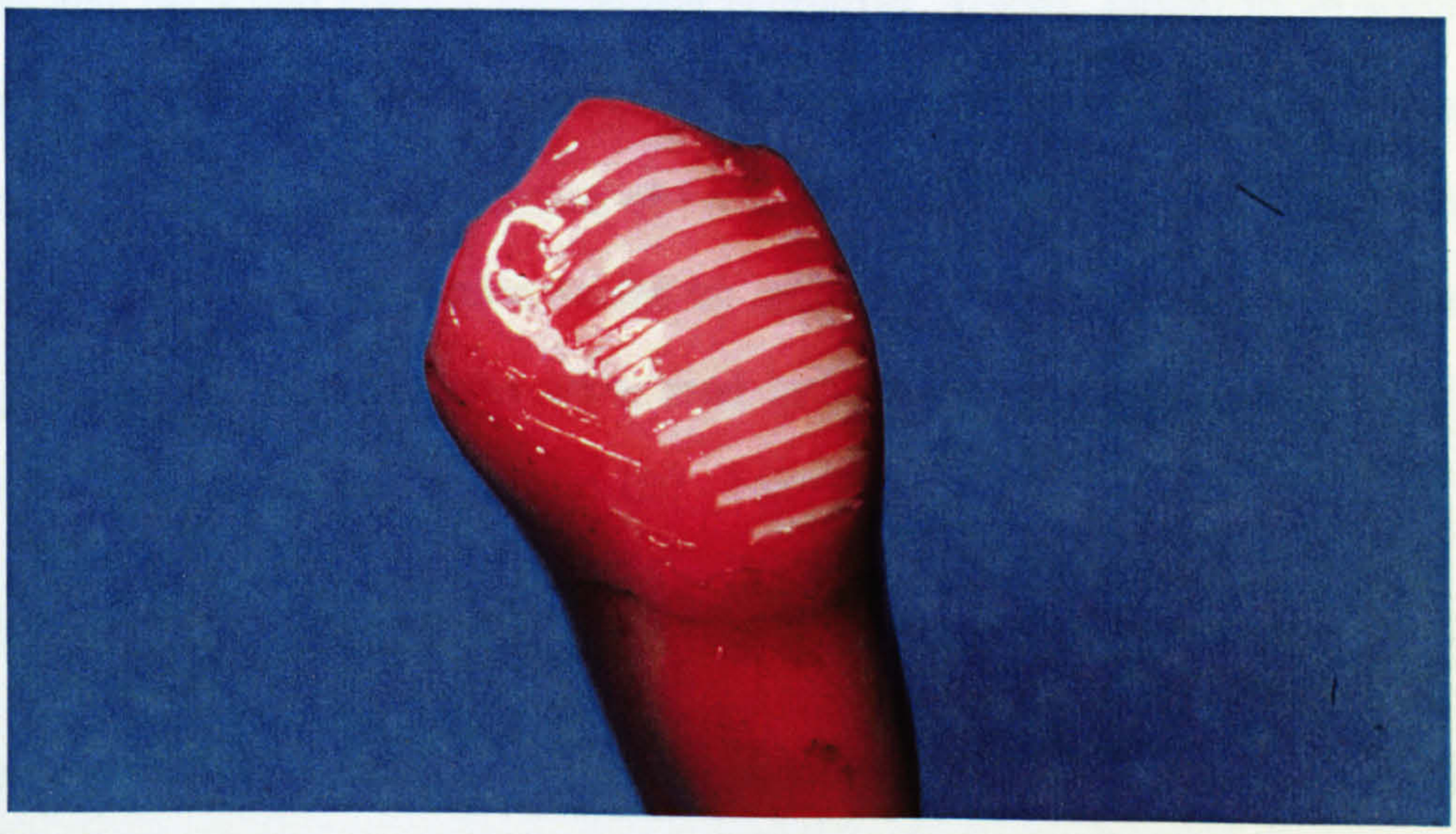
5.5.4 Section Preparation.

Following lesion creation, the roots were removed from each tooth, and the crowns were subsequently sectioned mesio-distally down the major fissure, using a dental drill (*Milbro*, Epson, England) and a diamond disc. The buccal half of the crown, containing the enamel lesions, was then mounted on to an acrylic block using a cyanoacrylate adhesive.

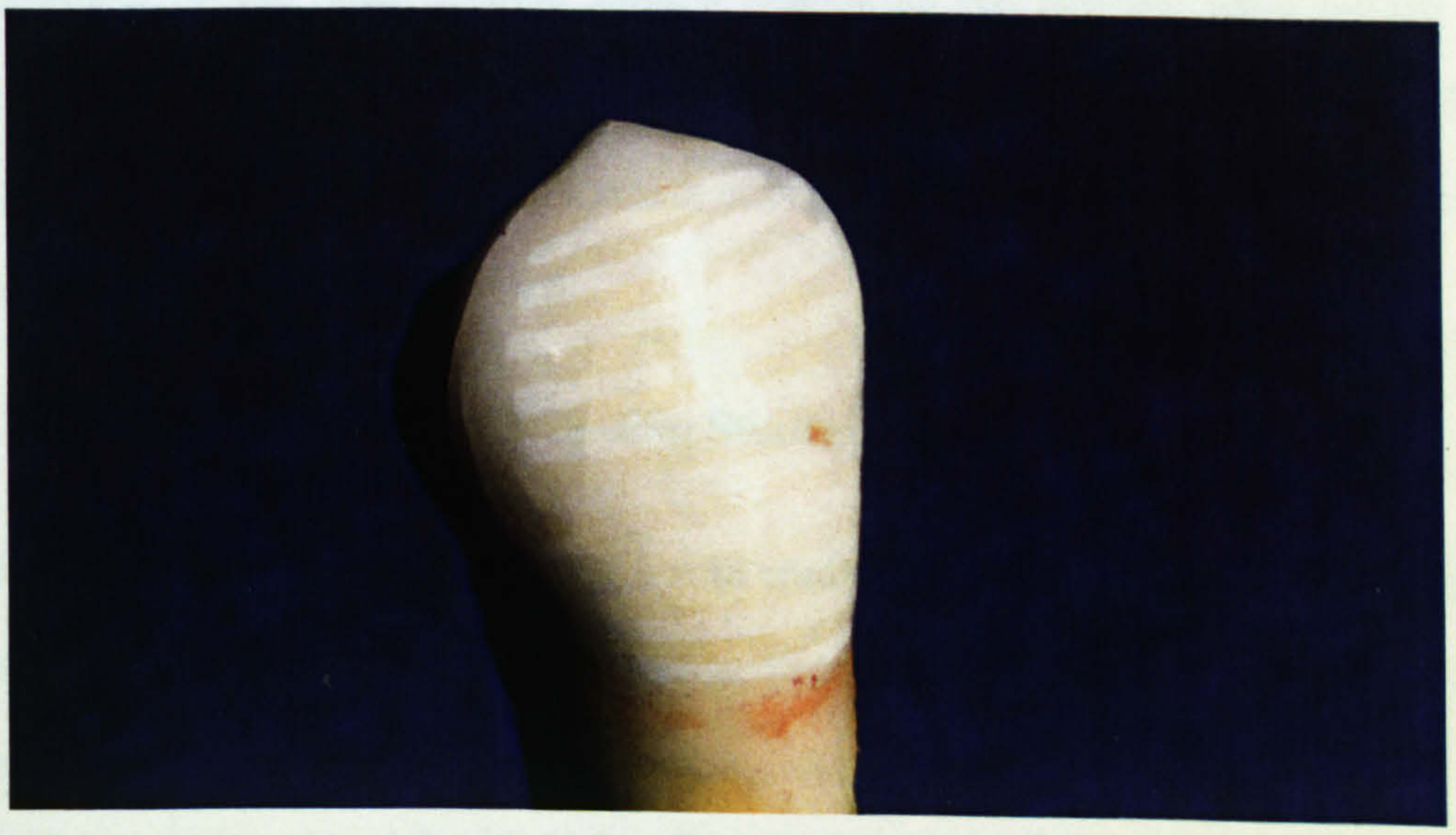
(* pI = ion activity product)

Figure 5.1 **Photographs of (a) varnished premolar tooth showing windows to allow artificial lesion creation and (b) human premolar tooth after varnish removal showing artificial caries leions.**

(a).



(b)



Sections were cut from this tooth block using the following standardised technique: sections were cut initially to a thickness of 250 μm with a circular diamond saw microtome (*E. Leitz Instrumental Ltd,* Luton, England), operated at minimal advancing speed and cooled by running water. These sections were then hand-ground to a plano-parallel finish on a glass plate using aluminium oxide or silicon carbide as an abrasive. The final thickness of the sections was measured using a micrometer *(Mitutoyo*, Tokyo, Japan) to an accuracy of 1 μ m. For each section, five measurements of the thickness were taken from the incisal to the cervical ends, and the mean thickness of each section calculated. In any instances where the mean thickness along the section varied by more than 5 μm, the thickness specific values corresponding to the lesion site were used in subsequent analysis. The range of section thickness used in this study was from 100 - 145 μm.

5.5.5 Varnishing Sections.

Before exposing the sections to any experimental protocol, all aspects of the specimens, with the exception of the outer enamel surface containing the lesions, were coated with acid-resistant varnish. This was carried out under a stereo-microscope at x 10 magnification and great care was taken to ensure the varnish covered all cut surfaces of the specimen, without encroaching on the artificial lesions.

5.4.6 Solutions for pH-Cycling Studies.

The demineralising solution used as the acid challenge was prepared using the same method as the lesion-creating solution described in Section 5.4.3, except that it contained 2 mM calcium chloride, 2 mM sodium dihydrogen orthophosphate and 50 mM glacial acetic acid. The pH was adjusted to 4.8 with 1M sodium hydroxide and fluoride added as NaF in the appropriate concentrations.

The remineralising solution, or artificial saliva, contained 2 mM dihydrogen orthophosphate and 2 mM calcium chloride at pH 6.85, and four batches of solution were prepared. Fluoride

was added in the form of NaF, at the appropriate concentrations for the experimental protocol i.e. <0.01, 0.02, 0.04 and 0.06 ppm F. These solutions were stable for approximately 24 hours, after which time precipitation was generally evident. To compensate for the metastable nature of the artificial saliva, stock solutions were prepared, one containing 4 mM calcium chloride and 4 mM sodium dihydrogen orthophosphate, and the other containing 2 mM sodium hydroxide. Prior to use, equal volumes of the two solutions were mixed, and the pH and fluoride concentration checked to ensure it was at the required value.

5.6 Microradiography/Microdensitometry.

5.6.1 Introduction.

As discussed in Section 5.3.1, quantitative microradiography and microdensitometry provide possibly the most sensitive and non-destructive techniques currently available for the determination of mineral changes in dental enamel (Angmar, Carlström & Glas,1963). This method of assessment has been quoted extensively in the literature (Groeneveld, Theuns & Kalter,1978; ten Cate & Duijsters,1982; Theuns *et al.*1983; Theuns *et al.*1984a; Theuns *et al.*1984b; Mallon & Mellberg,1985; Theuns, Driessens & van Dijk,1986; Strang *et al.*1987) and was the method of analysis used in the study reported in this thesis.

5.6.2 Microradiographic Methods.

Enamel sections of known thickness were mounted along with an aluminium step-wedge, on Kodak high resolution plates, Type 1A (Eastman Kodak Company, Rochester, New York, USA). These were then placed in light-tight holders and exposed to Cu(K_α) radiation from a Marconi X-ray tube (TX12) in an Enraf Nonius generator, operating at 20 kV and 30 mA, for 20 minutes. The focus-specimen distance was standardised at 300 mm. Previous studies (Creanor,1987; Damato,1990) have indicated that the X-ray beam is non-homogeneous in a

"north to south" direction. However, the beam variation in a horizontal direction was < 1%, and for this reason the calibrating aluminium step-wedge (thickness range 50-300 μm) was placed along the Y-axis of the plate. The radiation exposure parameters and the section thickness were selected to optimise the grey level range for the subsequent microdensitometric analysis. The radiographic plates were developed according to standard techniques.

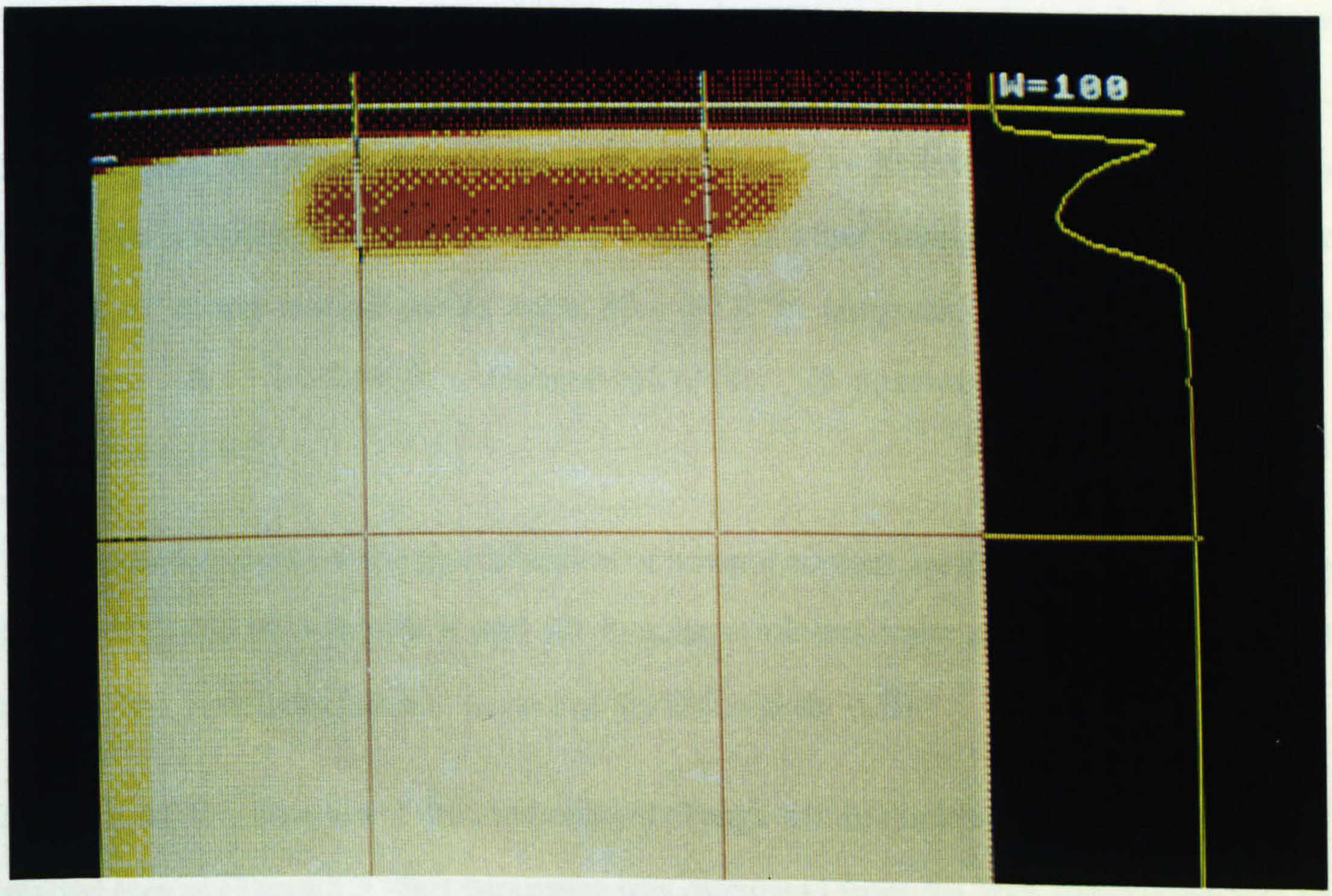
5.6.3 Microdensitometric Methods.

The microdensitometric apparatus used in this study was based on a *Leitz* ASBA Image Analyser. This consisted of a microscope (*Leitz* Dialux 22) with a stabilised power supply, a black and white video camera *(ASACA Corporation* Type 700BE), and a computerised image analyser (Z8002 microprocessor), which digitised the video signals from the camera. The digitised signals were transferred to a BBC microcomputer (*Acorn,* Cambridge, England) for subsequent analysis. The software for both the ASBA and the BBC microcomputer was written by Dr. R. Strang and Mr I. P. A. MacDonald (previously of the Dept. of Clinical Physics and Bioengineering, West of Scotland Health Boards).

Before each lesion was analysed, a calibration procedure was carried out. Firstly, the grey level of each of the aluminium step-wedge images (x 5 thicknesses; range 50-300 μm) on the microradiographic plate was recorded, and a fourth order polynomial calibration curve fitted to the data. In each instance, the area of the step-wedge image lying in the corresponding horizontal plane as the artificial lesion to be analysed was selected, in order to minimise any variation in the X-ray beam. The image of the relevant artificial lesion was then positioned under the microscope, and digitised into 256 x 256 pixels (1 pixel = 3 μm). The digitised data were then transferred to the BBC microcomputer for analysis.

The image of each lesion was displayed on the computer monitor and a suitable area of the lesion delineated as shown by the vertical lines on Figure 5.2. The average

Figure 5.2 **Photograph of the BBC microcomputer screen, showing the digitised image of an artificial caries lesion in enamel, with the area to be analysed delineated.**



microdensitometric profile within the area was then calculated in terms of grey levels (optical density). These measurements were then converted to % volume mineral content, using the equation derived by Angmar, Carlstrom & Glas (1963). The data were stored on a floppy disc and a hard copy of the lesion image, with its outlined "area of interest", was obtained using a "frame grabber" (Video Graphic Printer UP-701, *Sony*, Japan). This enabled the area of interest in each lesion to be repositioned accurately in subsequent radiographs.

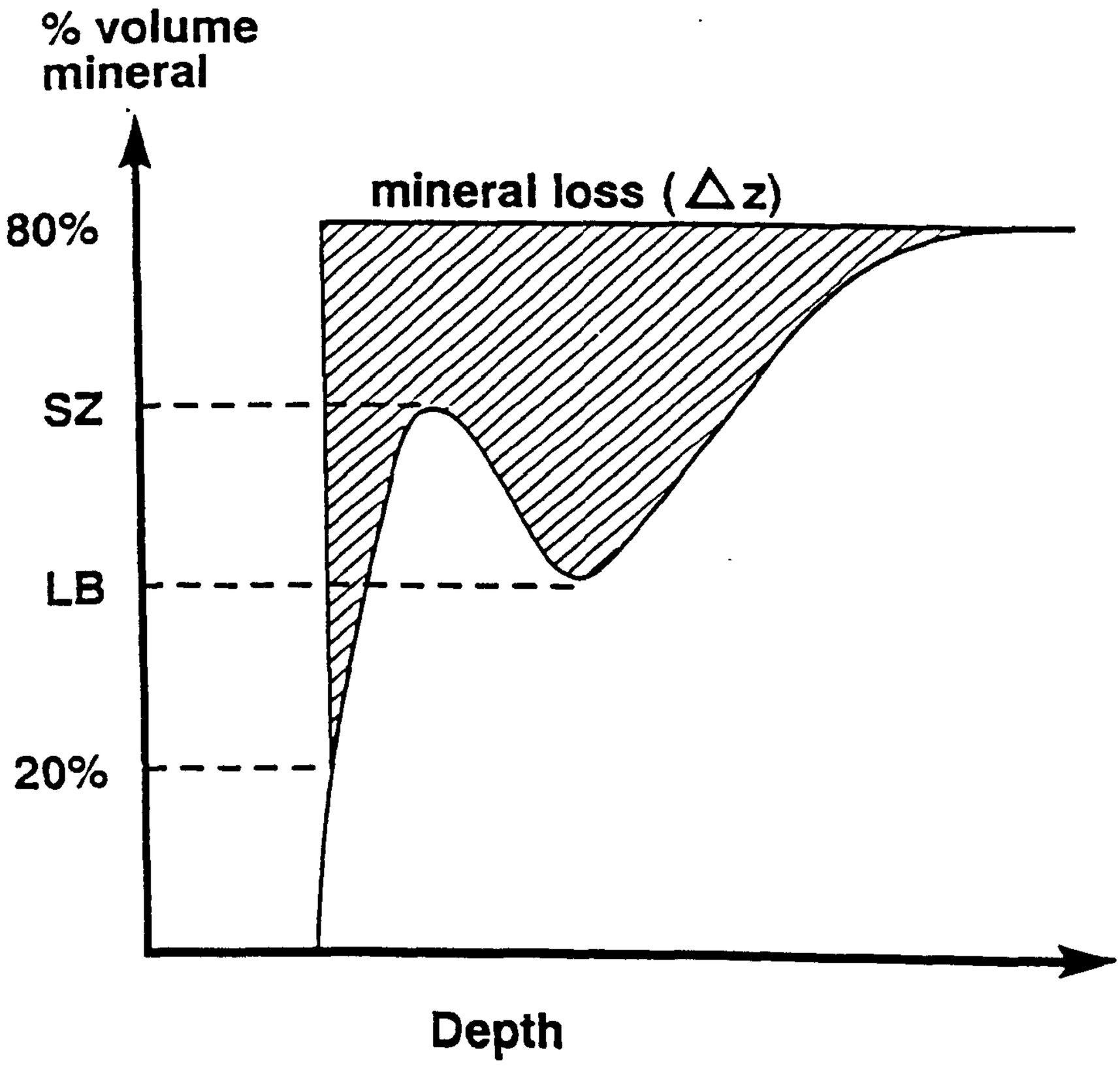
5.6.4 Microdensitometric Analysis.

Microdensitometric profiles of the lesions under investigation were analysed using software written in-house. A schematic representation of a lesion profile is included as Figure 5.3. The maximum % volume mineral was arbitrarily taken as 80% (Strang *et al.*1987) and all data were normalised accordingly. In a number of other studies the % volume mineral content of sound enamel varied from 82 - 87.2 % (Angmar, Carlström & Glas,1963; Bergman & Lind,1966; Groeneveld,1974; Hoppenbrouwers, Driessens & Borggreven,1986).

The parameters used for the quantification of mineral content are shown in Figure 5.3. These included: (i) ΔZ (% vol. min. x μm) (ii) % volume mineral content of the surface zone (SZ) and (iii) the % volume mineral content of the lesion body (LB).

Here, (i) ΔZ is a measure of the total mineral change within the lesion. It is denoted by the shaded area in Figure 5.3. This parameter was calculated from the 20% volume mineral on the initial slope of the microdensitometric profile, to a point "S" on the sound enamel. Many different definitions of ΔZ appear in the literature, some starting at 0% on the initial slope (Arends & ten Bosch,1986; Dijkman, Schultof & Arends,1986), and others at the surface zone maximum (Arends & Gelhard,1983; Mallon & Mellberg,1985; de Bruyn *et al.*1988; Leach, Lee & Edgar,1989). In this project, as in Creanor (1987) and Damato (1990), the

Figure 5.3 **Schematic representation of lesion mineral profile, illustrating the % volume mineral of the lesion with depth. ΔZ is the total mineral loss represented by the shaded area. SZ is the % volume mineral of the surface zone. LB is the % volume mineral of the lesion body.**



calculation of ΔZ commenced at 20% because it was found that values close to zero gave high errors due to the flattening out of the calibration curve.

When analysing the baseline mineral content, point "S" was selected visually by the operator, and had to lie within sound enamel. In subsequent analyses of the same lesion, point "S" was determined automatically by the computer, so that the distance between the 20% volume mineral point and point "S" was constant. Where a lesion extended beyond the original point "S", the baseline lesion was re-analysed, selecting a new point "S" further away from the lesion. Subsequent analyses were also carried out using this new point.

With respect to (ii), the volume % mineral content (subsequently referred to as % vol. min.) of the surface zone (SZ) is taken as the volume % mineral content of the maximum point in the microdensitometric profile (Fig. 5.3), with high values indicating a well-mineralised surface layer.

Finally, (iii), the lesion body value (LB) is the % vol. min. of the minimum point in Figure 5.3. Usually, substantial quantities of mineral are lost or gained from this region.

5.7 Results.

5.7.1 Introduction.

The full data set for ΔZ, SZ and LB parameters for this *in vitro* study are presented in Tables 5.1 to 5.4 for Group A (non F), Group B (0.02 ppm F), Group C (0.04 ppm F) and Group D (0.06 ppm F) respectively. For each lesion, a ΔZ, SZ and LB value was calculated at Weeks 0, 1, 2, 3, 4 & 5, and therefore the overall changes quoted in the analysis are calculated from the slope of the regression line, unless otherwise stated. A straight line was drawn from the data using a least squares technique, and its slope taken as a measure of the de-/remineralisation rate for that lesion and for that parameter. The standard errors (SE) of the individual lesions were derived from the computed variance for the slope of the

Table 5.1 **Group A (non-F re- and demineralising solutions) ΔZ, SZ and LB for all lesions at each weekly time-point.**

		Baseline			Week 1			Week 2			Week 3			Week 4			Week 5		
	LESION	ΔZ	SZ	LB	ΔZ	SZ	LB	ΔZ	SZ	LB	ΔZ	SZ	LB	ΔZ	SZ	LB	ΔZ	SZ	LB
1	DIA13	2888	60.42	57.94	3179	54.83	54.83	3315	54.58	57.27	3338	54.79	51.65	3231	54.89	49.88	3371	50.92	49.74
2	DIA14	6061	45.23	41.63	5596	44.70	32.18	5281	47.48	35.72	5800	46.37	33.39	5596	46.51	34.20	5187	41.68	34.08
3	DIA31	1456	60.66	49.19	2234	50.82	41.62	2244	51.85	40.18	2212	52.75	42.93	2271	51.86	41.63	1864	57.87	46.96
4	DIA32	2208	71.25	52.07	2897	62.11	41.16	2927	60.57	40.00	2626	61.03	44.30	2869	58.37	41.33	2771	55.46	43.14
5	DIA33	1547	69.99	61.98	2346	65.75	52.91	2269	65.74	54.77	2122	67.31	55.75	2360	65.47	52.60	1777	68.36	56.56
6	DIA34	2375	70.25	47.42	3080	64.64	38.84	2592	67.87	42.43	3064	60.56	41.56	3274	59.08	40.07	2641	61.08	42.28
7	DIA42	1952	67.65	48.27	3625	63.31	32.98	3896	57.42	32.80	3974	58.27	32.40	4206	41.20	27.14	4258	43.41	28.48
8	DIA62	3098	67.58	45.39	4963	59.79	34.07	4874	56.98	36.94	4836	58.26	37.02	4863	54.05	35.51	4470	52.16	37.63
9	DIA63	1903	72.10	57.91	3632	62.25	40.44	3626	60.12	41.79	3549	60.29	43.90	3572	56.59	42.47	3201	52.59	43.79
10	DIA64	2836	70.53	54.68	3286	65.02	42.24	3464	63.00	43.49	3420	62.70	43.66	3515	58.13	43.02	3808	52.67	39.94
11	DIA72	3183	62.55	36.31	4295	57.33	29.49	4348	61.37	33.79	4366	56.35	33.10	4012	59.36	35.10	3459	61.02	37.61
12	DIA73	1845	66.91	59.82	2536	63.17	53.52	2218	68.64	54.16	2485	64.53	54.90	2280	63.59	52.18	2088	61.46	56.13
13	DIA74	2426	63.87	55.23	2860	58.17	50.12	2681	56.37	51.96	2510	59.80	51.51	2412	61.45	51.34	2326	63.45	50.92
14	DIA82	2878	51.89	45.44	3600	49.26	43.96	3397	53.43	43.12	3828	45.63	38.79	3861	46.75	39.08	3095	50.29	45.18
15	DIA83	2686	60.53	53.89	3210	55.61	50.15	3057	59.55	45.55	3392	55.21	50.38	3241	56.07	48.00	3242	50.40	45.38
16	DIAX1	4279	53.24	35.48	5700	46.73	24.85	5910	44.88	26.82	5782	47.84	29.05	6492	31.10	19.71	61.02	26.44	19.02
17	DIAX2	2364	67.17	45.88	3222	66.72	38.08	3366	62.49	40.85	3268	61.87	41.98	3449	61.48	40.97	3265	59.31	43.11
18	DIAX3	1649	70.60	55.48	2350	67.81	46.15	2288	68.52	51.27	2710	65.25	47.73	2571	65.37	48.28	2705	59.12	46.90

Table 5.2 **Group B (0.02 ppm F re- and demineralising solutions) ΔZ, SZ and LB for all lesions at each weekly time-point.**

	Baseline			Week 1			Week 2			Week 3			Week 4			Week 5		
LESION	ΔZ	SZ	LB	ΔZ	SZ	LB	ΔZ	SZ	LB	ΔZ	SZ	LB	ΔZ	SZ	LB	ΔZ	SZ	LB
1 DIB12	3110	55.94	51.48	3419	52.96	49.49	4629	44.18	42.12	4050	49.40	47.24	3924	49.75	48.18	3391	51.36	49.23
2 DIB13	2950	60.04	53.36	3510	57.73	48.03	4069	52.22	45.59	3854	54.78	47.36	3894	53.34	46.93	3528	52.27	44.38
3 DIB14	3683	55.58	46.21	3893	56.80	43.77	3927	54.59	46.63	3726	55.59	47.73	3999	53.79	44.02	3753	54.06	42.05
4 DIB21	1512	65.59	57.93	2018	58.97	46.66	2671	51.29	42.11	2470	53.96	45.58	2264	54.14	45.23	1708	65.01	52.54
5 DIB22	1883	65.02	59.13	2157	62.78	53.38	1949	65.63	57.49	1924	63.50	55.27	2191	62.74	55.29	1506	68.64	59.27
6 DIB23	3602	53.95	40.56	4104	54.52	34.74	4367	50.65	34.65	3937	53.55	35.83	3891	53.10	36.58	3866	52.86	37.83
7 DIB32	2568	60.99	56.99	3242	55.68	49.10	3604	55.35	49.62	3535	54.93	48.58	3388	56.94	51.55	3189	57.74	49.46
8 DIB33	4414	45.59	41.54	5460	45.00	35.02	5592	46.32	36.20	5763	43.09	33.11	5956	44.25	34.09	5549	40.94	30.27
9 DIB51	1902	65.32	60.15	2528	60.87	51.05	2711	60.84	50.83	2608	60.35	49.07	2298	64.47	55.19	3124	54.18	44.86
10 DIB52	3188	60.25	42.93	3483	62.48	38.02	3902	59.29	37.39	3762	61.56	37.20	3723	63.54	37.98	3293	61.10	40.82
11 DIB53	3953	56.01	39.65	4482	51.87	34.54	4612	53.35	35.28	4612	54.53	34.99	4604	56.28	35.41	4012	57.45	37.96
12 DIB62	4106	60.13	39.87	4688	62.57	37.87	5043	58.20	38.25	4662	60.43	40.01	4751	59.06	39.42	6490	55.02	37.21
13 DIB63	2629	68.52	49.59	3007	67.73	45.95	3151	65.73	46.92	2987	67.24	47.73	2607	69.41	53.86	2932	63.62	46.24
14 DIB64	2420	67.86	46.53	2786	70.31	44.79	2849	69.63	45.68	2663	71.16	49.00	2638	71.79	48.50	1857	74.95	53.83
15 DIB71	2958	59.24	53.50	4062	49.05	32.04	4091	50.30	34.05	4450	46.61	30.55	4506	44.95	30.87	3578	46.61	37.49
16 DIB72	1655	67.57	56.69	2171	64.16	50.29	2060	67.80	52.06	2151	69.57	51.32	2045	70.34	53.18	1877	70.73	53.07
17 DIB82	2185	67.17	51.37	2955	65.45	45.79	2541	69.28	51.32	2702	67.79	48.69	2491	69.32	51.54	3046	64.04	42.14
18 DIB83	1942	67.50	56.85	2214	72.27	56.17	2144	71.26	55.01	2150	71.59	54.60	2072	73.88	58.01	2016	68.80	60.70
19 DIB84	2554	63.05	56.29	3355	59.86	51.16	3283	58.22	49.96	3562	56.04	47.43	3605	55.63	47.05	3037	54.72	51.54
20 DIB92	2701	68.03	41.83	3974	65.44	32.87	3619	66.17	34.89	3852	63.67	31.46	3661	66.75	34.47	2651	67.19	45.63
21 DIB93	2301	66.30	44.01	2795	66.82	41.49	3015	64.27	41.56	2870	66.17	42.96	2643	68.20	45.34	2442	63.88	47.50

Table 5.3 Group C (0.04 ppm F re- and demineralising solutions) ΔZ, SZ and LB for all lesions at each weekly time-point.

		Baseline			Week 1			Week 2			Week 3			Week 4			Week 5		
	LESION	ΔZ	SZ	LB	ΔZ	SZ	LB	ΔZ	SZ	LB	ΔZ	SZ	LB	ΔZ	SZ	LB	ΔZ	SZ	LB
1	DIC11	4553	44.91	39.85	5172	48.19	36.30	5131	49.14	35.58	5429	47.81	33.08	5828	47.78	32.76	5806	43.93	31.36
2	DIC12	2376	62.70	53.08	3543	56.83	43.94	3511	58.25	41.52	3523	59.34	40.35	3560	59.61	41.51	3551	59.93	40.03
3	DIC13	3693	55.30	42.42	4731	49.71	35.42	4996	51.13	34.82	5367	47.21	30.74	5745	45.69	28.23	6269	36.80	25.06
4	DIC21	2502	58.85	49.49	2923	61.97	43.29	2739	60.29	46.38	2796	61.98	44.96	3151	55.68	42.24	3156	49.26	38.93
5	DIC51	5955	53.87	43.22	6356	49.78	29.91	5575	54.85	31.86	6191	50.60	27.42	6063	52.51	28.41	4952	57.61	32.60
6	DIC52	3764	52.92	42.57	4416	51.12	36.70	3965	56.92	37.70	4196	56.51	36.19	4130	55.23	34.21	3768	55.16	35.86
7	DIC54	4448	47.15	39.11	5014	48.60	34.50	4537	53.62	36.44	4561	55.86	34.33	4582	55.66	35.82	4252	56.46	34.18
8	DIC61	3513	54.57	48.77	3790	52.40	46.14	3696	57.30	47.71	4011	53.23	44.05	4038	53.75	44.32	3825	55.59	47.07
9	DIC62	4586	47.98	43.87	5656	49.15	40.07	4910	53.52	43.28	4856	54.10	42.56	5061	55.67	41.37	5087	49.01	39.74
10	DIC63	3016	58.07	54.24	3815	52.32	46.63	3553	57.91	49.69	3502	57.95	50.63	3633	58.33	47.36	3360	56.86	50.29
11	DIC71	3130	68.42	47.39	4395	61.04	31.55	4376	65.39	36.96	3130	68.42	47.39	4395	61.04	31.55	4376	65.39	36.96
12	DIC72	2603	71.27	50.92	3748	66.15	38.96	3337	70.42	45.71	3304	70.16	40.87	3347	69.42	41.26	2733	73.67	44.73
13	DIC73	2797	67.87	53.75	2610	66.50	42.34	3592	66.02	45.14	3259	69.12	41.04	3365	67.71	41.02	3473	66.10	39.19
14	DIC74	2811	64.88	56.22	3337	65.46	43.26	3605	20.33	18.99	2991	69.04	45.19	3019	68.59	46.08	2660	70.68	47.61
15	DICX1	3690	52.82	42.48	4077	54.45	39.91	4024	57.98	43.45	4266	57.72	40.44	3898	59.66	43.81	3478	59.77	45.86

Table 5.4 Group D (0.06 ppm F in re- and demineralising solutions) ΔZ, SZ and LB for all lesions at each weekly time-point.

		Baseline			Week 1			Week 2			Week 3			Week 4			Week 5		
	LESION	ΔZ	SZ	LB	ΔZ	SZ	LB	ΔZ	SZ	LB	ΔZ	SZ	LB	ΔZ	SZ	LB	ΔZ	SZ	LB
1	DID11	3438	52.48	35.83	4085	54.40	32.94	4039	51.24	34.95	3930	52.98	34.93	4535	48.81	29.93	3964	49.49	34.97
2	DID12	2272	61.48	50.02	3561	54.71	36.86	3624	54.15	37.68	3640	54.17	39.53	3803	54.34	36.62	3110	56.77	40.54
3	DID21	3013	47.07	41.27	3122	52.93	44.61	2831	60.21	44.51	2890	60.87	46.04	3203	56.38	41.91	2574	58.52	48.33
4	DID22	3296	52.00	45.60	3522	53.06	41.90	3142	57.48	40.55	3337	57.50	40.75	3397	55.43	38.31	3217	57.41	41.62
5	DID31	3562	59.22	42.22	3670	54.08	37.07	4092	44.87	37.39	4356	44.92	35.40	4718	39.21	31.48	3603	50.64	43.14
6	DID32	2982	64.07	52.00	2515	63.38	52.95	2719	62.80	51.00	2594	64.39	53.71	2781	61.60	49.14	1890	68.46	57.86
7	DID33	4423	58.03	39.95	4393	55.50	35.05	4681	52.98	35.29	5112	49.44	31.66	5583	46.03	25.89	4628	51.74	35.59
8	DID42	3617	66.08	39.52	3017	65.60	41.86	3291	64.06	39.83	3217	64.31	41.38	3629	62.28	37.30	2937	63.58	43.15
9	DID52	3118	65.02	51.42	3799	57.24	42.80	3283	63.48	45.17	3604	62.29	44.83	3602	62.99	42.23	3192	58.88	46.83
10	DID53	1484	70.83	60.96	1673	69.47	56.79	1582	69.25	59.41	1687	68.86	59.58	1513	70.24	58.97	1619	65.98	57.46
11	DID54	2729	70.54	53.30	2518	68.44	53.30	2684	68.35	51.93	2824	68.33	51.25	2968	68.51	48.04	3283	65.04	48.67
12	DID72	3380	61.37	40.00	3003	60.96	37.52	3243	62.36	33.89	2949	64.66	43.36	3567	58.96	31.94	2846	64.26	40.95
13	DID73	1988	70.96	48.77	2252	65.71	39.10	2220	69.90	41.02	2148	70.16	41.38	2270	71.45	37.32	1559	73.34	51.53
14	DID82	3050	64.00	53.35	2610	63.82	51.32	2835	63.08	51.41	3046	61.12	48.79	2821	63.21	49.53	2797	59.66	50.13
15	DID83	2729	64.57	54.65	2809	63.15	51.02	3068	61.52	49.26	2933	60.76	49.15	3104	61.58	48.25	2690	61.48	51.25
16	DID84	3013	60.62	52.78	2544	62.49	53.25	2775	60.77	51.93	2520	61.09	53.27	2506	60.83	50.80	2468	58.28	52.75
17	DID92	3475	68.86	40.46	3613	64.03	35.87	3664	64.51	36.45	3402	68.50	38.58	3549	67.09	35.57	3322	65.90	37.87
18	DID93	2980	69.10	42.43	3082	64.00	41.51	2768	66.10	40.97	2910	65.89	37.86	2739	67.19	42.23	2312	67.51	44.73
19	DIDX1	4881	50.46	45.80	4410	45.65	40.22	4289	47.24	43.10	4154	47.57	43.54	4719	36.96	35.15	3897	41.23	41.15
20	DIDX2	3735	57.80	50.91	3214	58.19	48.84	3103	59.11	52.88	3037	59.95	53.85	3222	61.83	54.54	2705	59.57	52.95

regression analysis (the standard error of the mean being used with the mean values reported throughout the results).

Only those lesions which had a complete data set are included in the analysis. Those data relating to lesions which were damaged during removal and replacement on the pH cycling machine, or which were lost completely (four lesions on one section in Group C) were discarded from any analysis. The loss of lesions due to damage occurred randomly throughout the four groups.

The results in this chapter are presented for ΔZ, SZ and LB over the following three sections. Each data set was plotted over time to illustrate the pattern of mineral change, and an overall mean, for each of the four lesion groups and each parameter, is also provided for illustrative purposes. As mentioned above, the slope of the regression line for each lesion was used in the analysis, as this is a more accurate reflection of the re-/demineralisation rate than simply subtracting the last values from the initial values. Subsequently, each data set was subjected to two types of Analysis of Variance. Firstly, ANOVA was undertaken to identify any significant differences between the four groups, for each of the ΔZ, SZ and LB parameters. Secondly, in order to allow for variation in baseline lesion mineral size, ANOVA on the slope of the regression line divided by the baseline lesion mineral size was undertaken. Where significant differences were identified with ANOVA, this was followed up by two-sample T-tests, to identify which groups were significantly different from each other.

5.7.2 Results for ΔZ.

The mean baseline, or initial, ΔZ for each group was 2646, 2775, 3562 and 3158 % vol.min. x μm for Group A, B, C and D respectively. A single factor analysis of variance (ANOVA) identified significant differences between the group baseline ΔZ values (Table 5.5, $p < 0.05$). Subsequent analysis using two-sample T-tests revealed the significant differences were between Groups A & C, and Groups B & C. This finding is discussed in detail in

Table 5.5 **Baseline ΔZ single factor Analysis of Variance: Groups A, B, C & D.**

Variable	Degrees of Freedom	Sum of Squares	Mean Square	F Statistic	p value
Between Groups	3	8550018	2850006	3.388	0.023
Within Groups	70	58879907	841141		
Total	73	67429925			

Section 5.8.

In Figures 5.4 - 5.7, the changes in ΔZ for each lesion in the four groups are plotted against time. For all data presented, an increase in ΔZ corresponds to a net demineralisation, whilst an increase in SZ or LB (% vol.min.) implies an increased mineral content, and therefore a net remineralisation.

Furthermore, Figure 5.8 illustrates the mean weekly change in ΔZ for all the lesions in each group (i.e. mean of week 1 minus week 0, mean of week 2 minus week 1 etc.). The data points lying on the y-axis are, therefore, the difference in ΔZ between week 1 and week 0 for each group, and demonstrate a net demineralisation of 832, 581, 676 and 12 % vol.min x μ m for Groups A, B, C and D respectively.

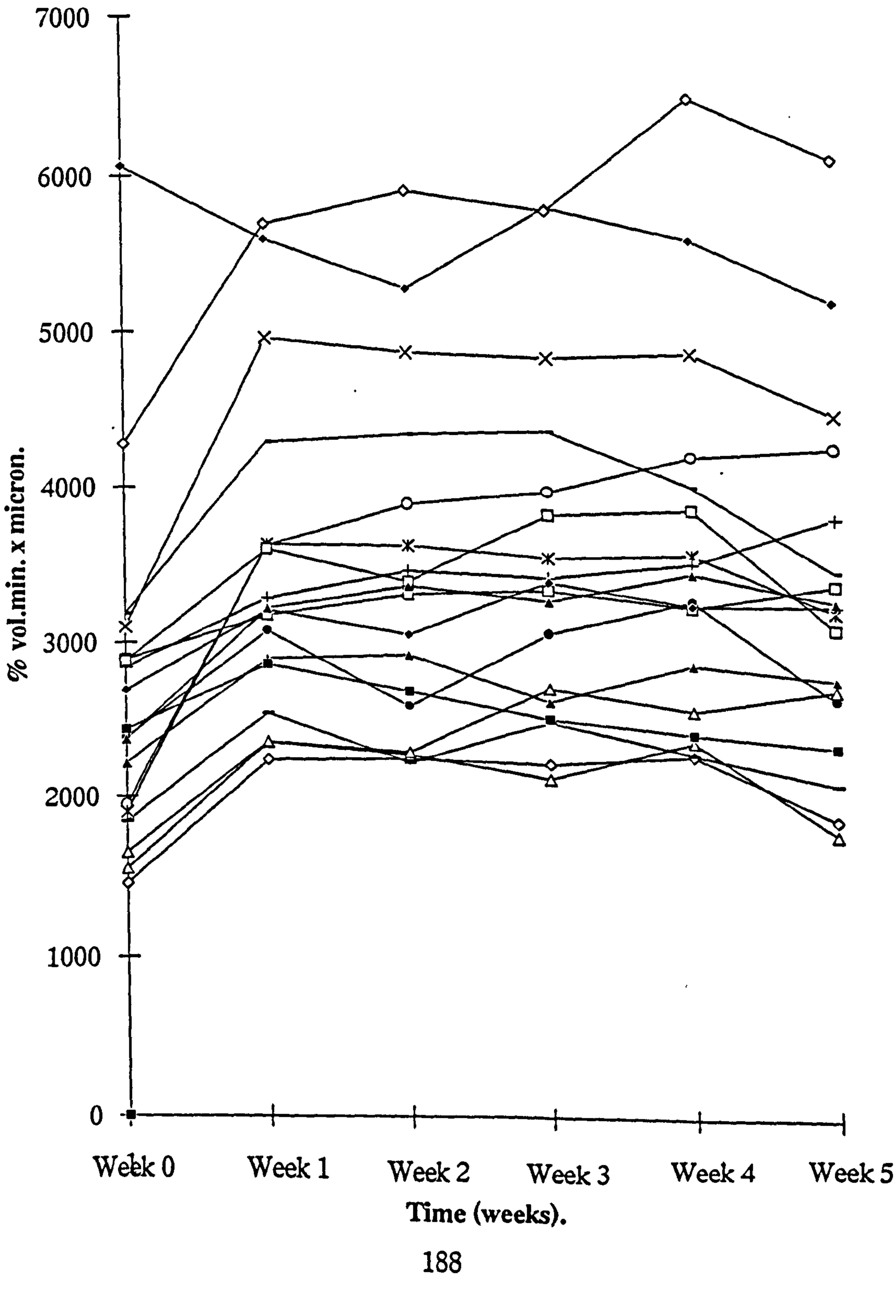
For Groups A, B and C, there is a suggestion of a cyclical pattern of change in ΔZ with time. For example, an extensive period of demineralisation during week 1 is followed by remineralisation during week 2, whilst the demineralisation which occurred in week 3 is followed by further remineralisation in week 4.

For Group D (0.06 ppm F), there is a very small net demineralisation over weeks 0-4, followed by a significant remineralisation, as reflected by the decrease in ΔZ, over the latter stages of the experiment.

Figure 5.9 is for illustrative purposes only, and shows the overall mean change in ΔZ, i.e. week 5 minus week 0, ignoring the intervening timepoints, for all four groups. The highest fluoride concentration (Group D) was the only group to show an overall increase in ΔZ and therefore a net remineralisation over the experimental period.

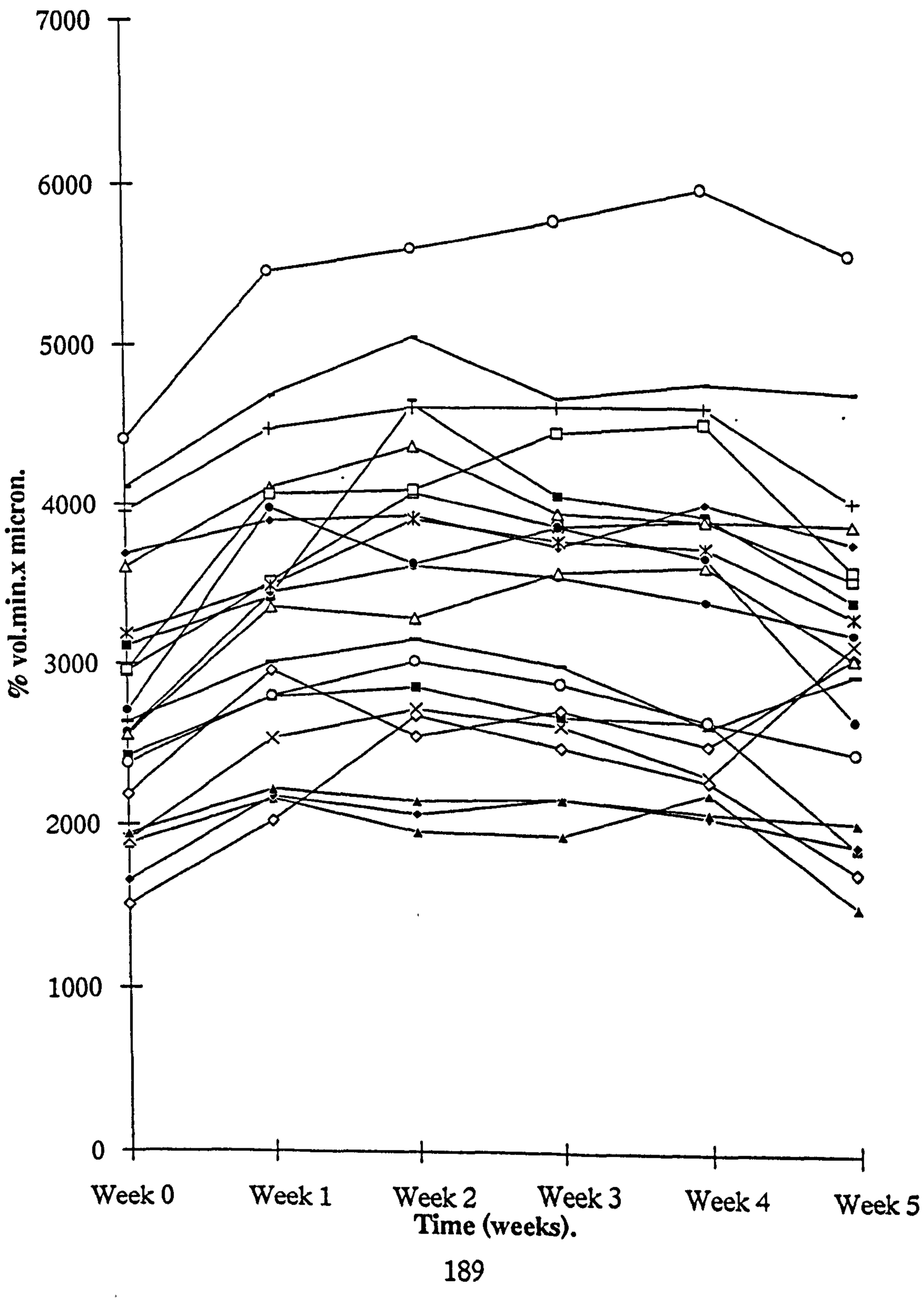
In Tables 5.6 and 5.7, the rate of ΔZ re-/demineralisation for each lesion is given. In Group A, for example, 16 of the 18 lesions have a positive slope for the regression line, which for Δ Z represents a demineralisation rate, whilst in Group D, 11 out of 20 lesions have a net

Figure 5.4 Group A: ΔZ (non F) versus time (Weeks) for 18 lesions.



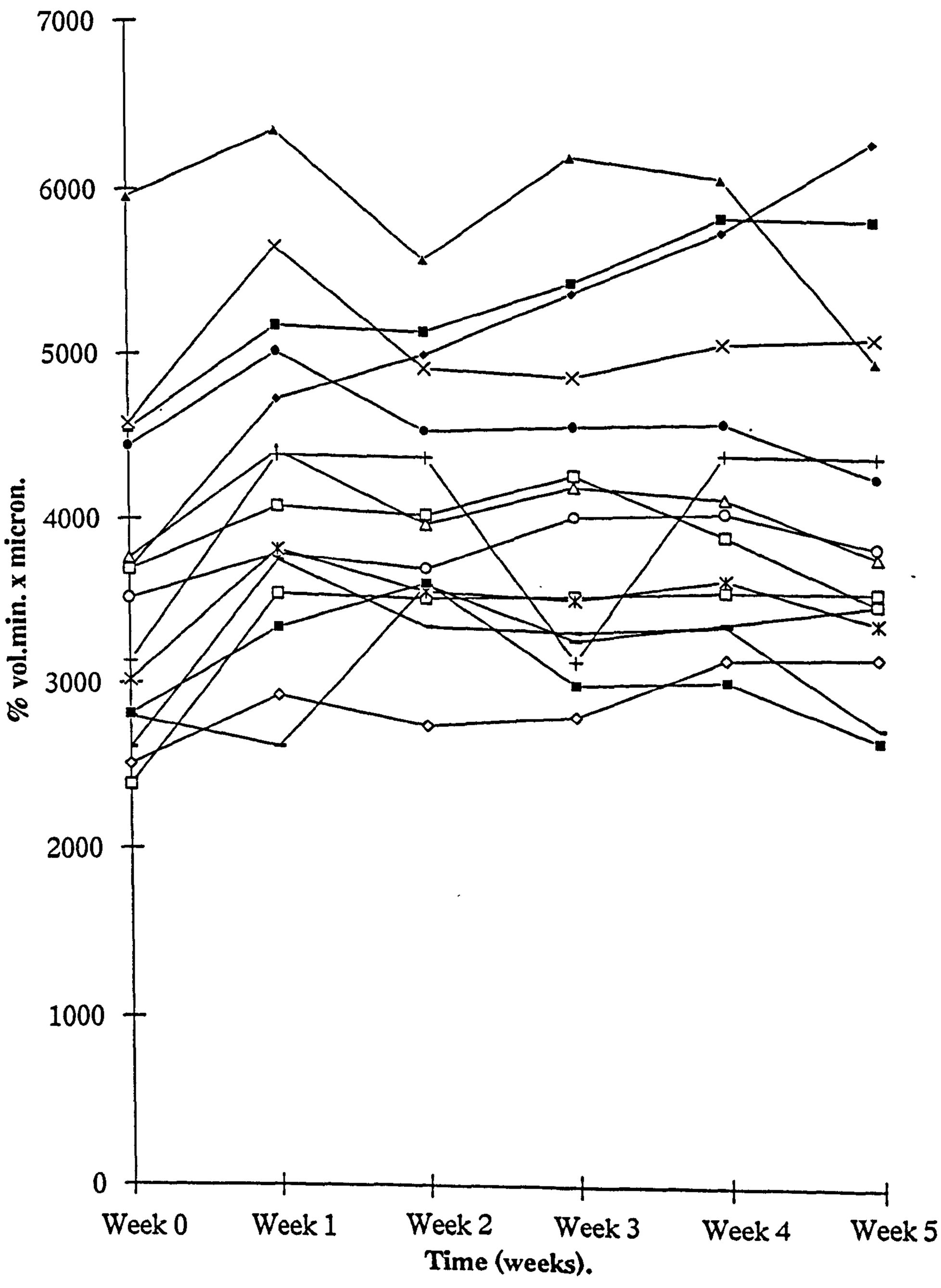
An increase in ΔZ equals net demineralisation.

Figure 5.5 Group B (0.02 ppm F)ΔZ versus time (Weeks) for 21 lesions.



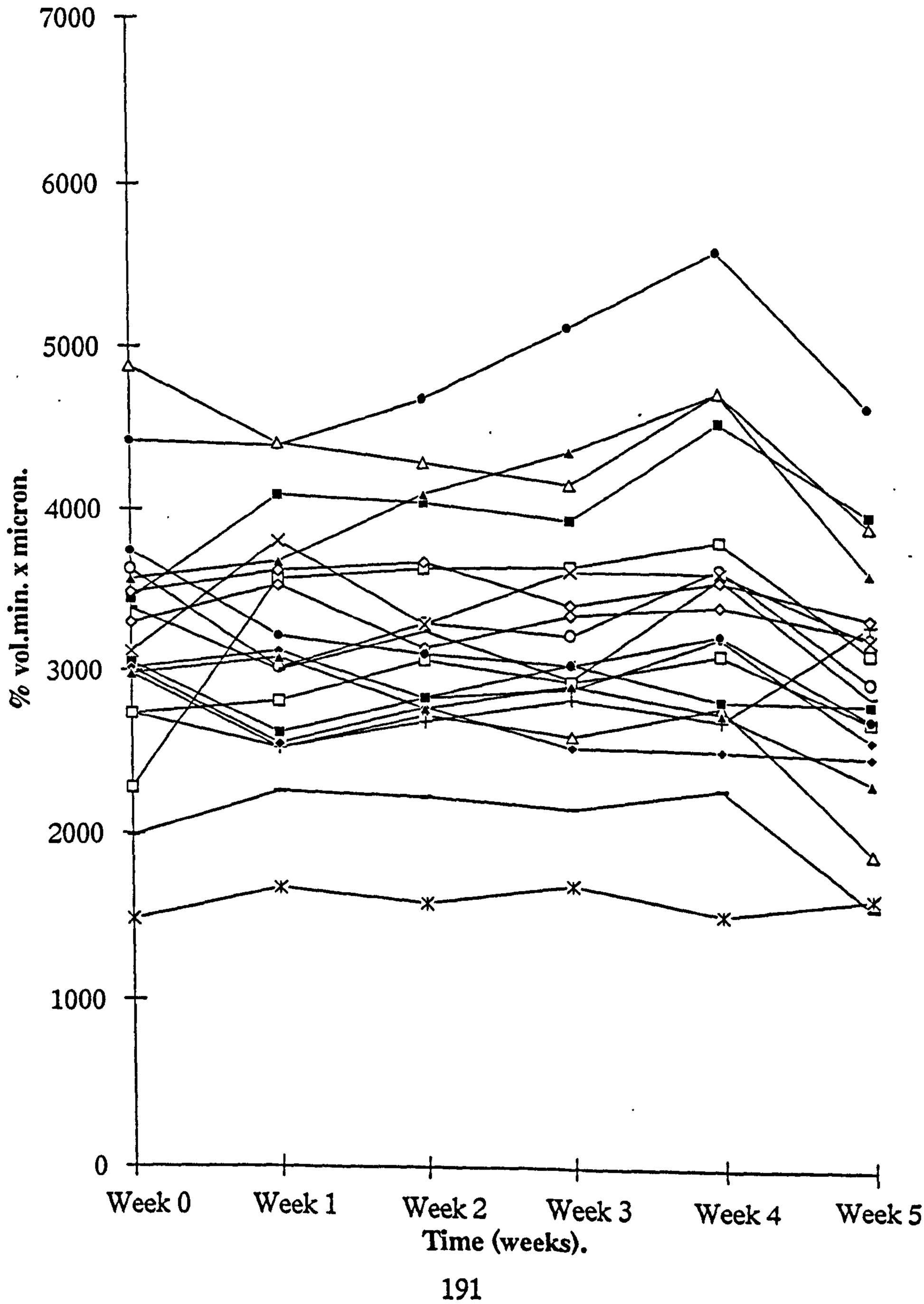
An increase inΔZ equals a net demineralisation.

Figure 5.6 Group C (0.04 ppm F)ΔZ versus time (Weeks) for 15 lesions.



An increase inΔZ equals a net demineralisation.

Figure 5.7 Group D (0.06 ppm F)ΔZ versus time (Weeks) for 20 lesions.



An increase inΔZ equals a net demineralisation.

Figure 5.8 Mean change inΔZ with time (weeks) for all lesions in each of Groups A, B, C & D.

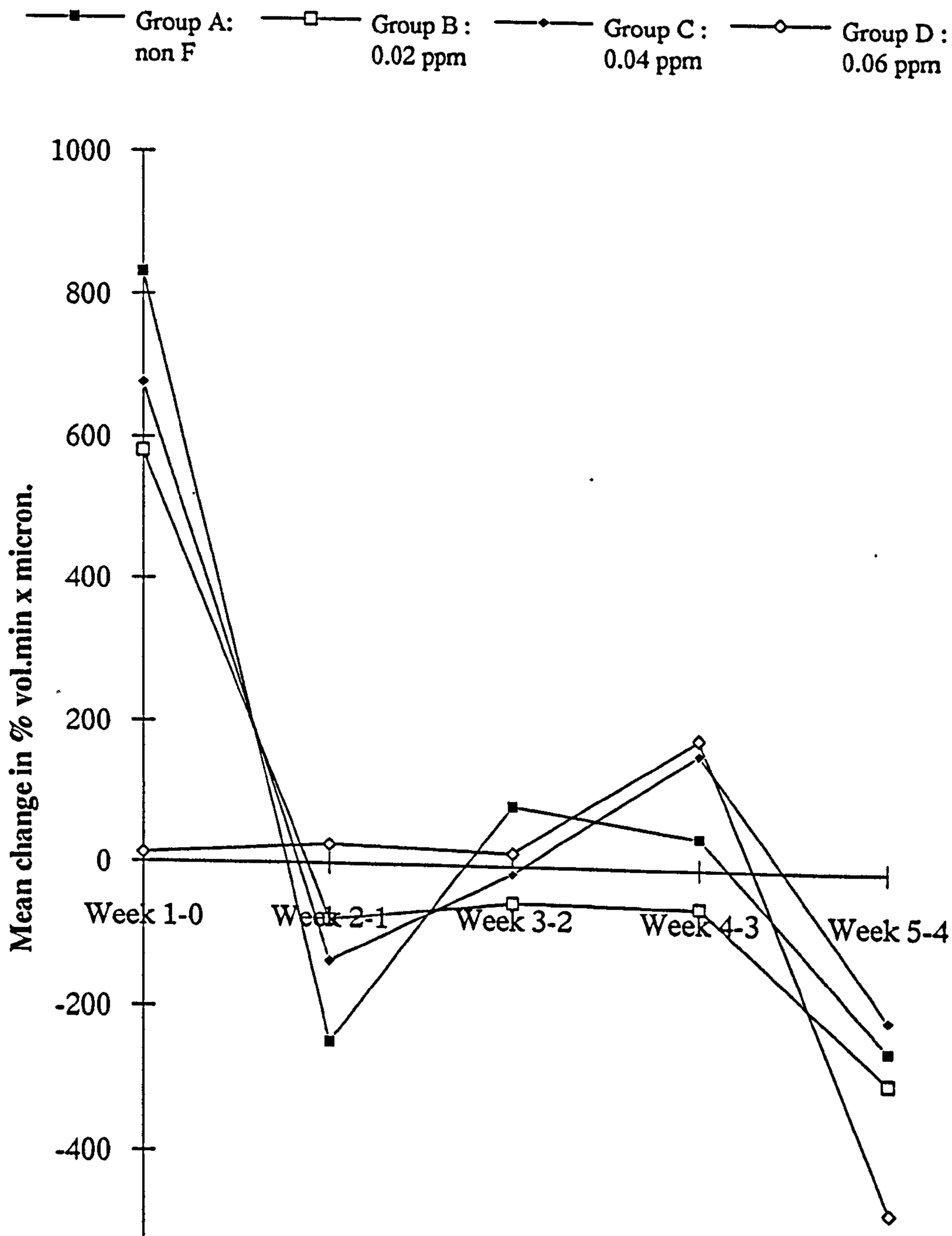
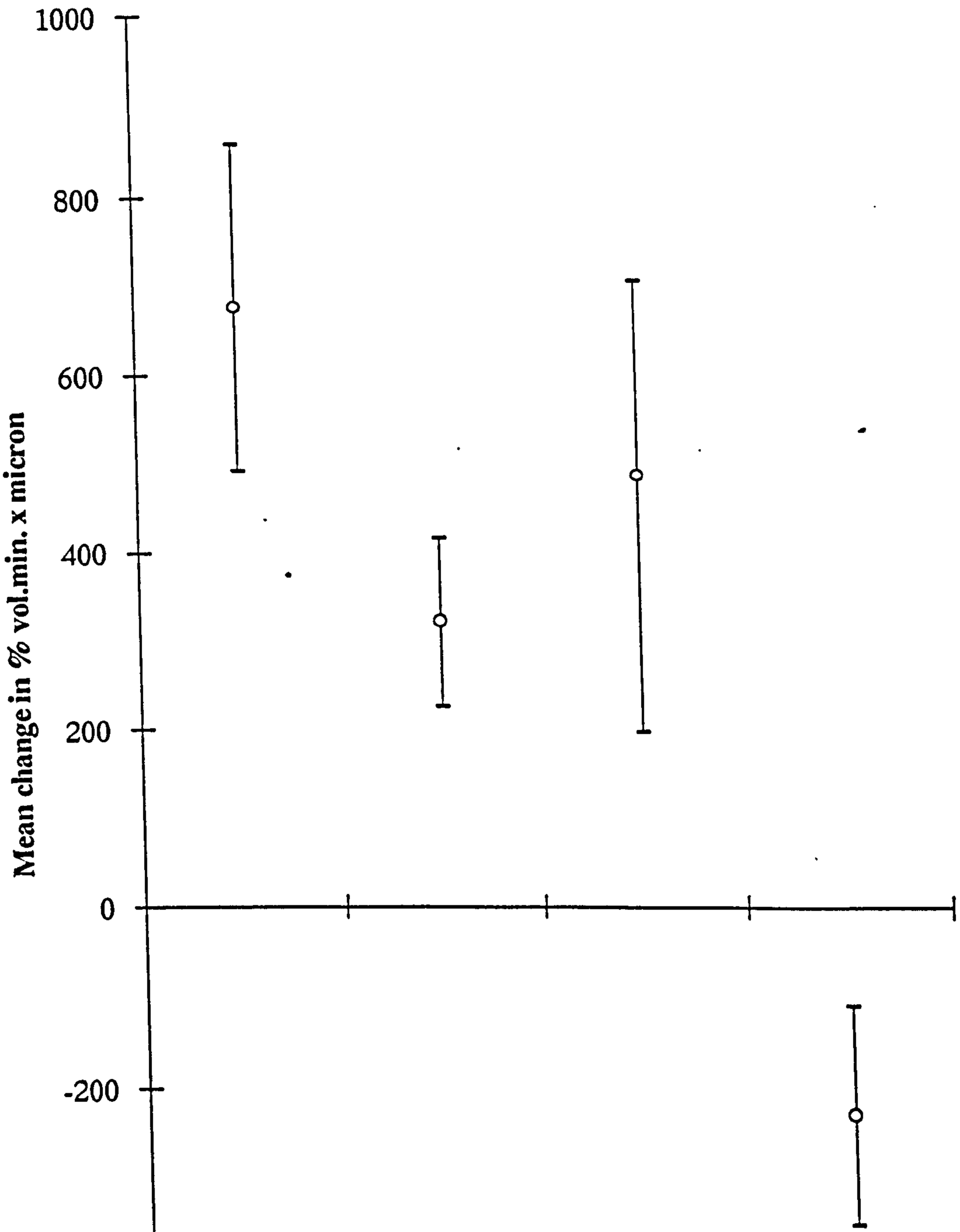


Figure 5.9 Mean (S.E.) forΔZ (week 5 minus week 0) for Groups A, B, C & D.



demineralisation rate.

To establish if there was any significant difference between the ΔZ re-/demineralisation rates, Analysis of Variance (ANOVA) was undertaken on the slope of the regression line for all lesions in each group. The results of this ANOVA are presented in Table 5.8, and show a borderline, non -significant difference ($p > 0.05$) in the rate of change in ΔZ between the groups.

In view of earlier work undertaken (Strang *et al.*1986), a subsequent ANOVA was conducted. In this instance, the slope of each regression line was divided by the initial ΔZ value, thus factoring in the variation in baseline lesion size. The result of this second ANOVA is presented in Table 5.9, and shows a borderline significant difference in re-/demineralisation rate between the groups ($p < 0.05$).

These ANOVA's were followed up by individual comparisons of each group with the three others, using a two-sample T-test, with a 99.17% Confidence Interval to compensate for the multiple comparisons (see Tables 5.18 and 5.19). The results of the follow-up analyses are discussed in Section 5.7.5 (Summary Statistics).

Table 5.6 **Rate of change in ΔZ for each lesion as calculated from the slope of the regression line: Group A (non F) & Group B (0.02ppm F).**

Group A (non F) lesion	Re-/demineralisation rate*	Group B (0.02 ppm F) lesion	Re-/demineralisation rate*
DIA13	+74.1	DIB12	+67.0
DIA14	+60.5	DIB13	+109.0
DIA31	+69.4	DIB14	+13.3
DIA32	+29.9	DIB21	+43.0
DIA33	+68.1	DIB22	-51.7
DIA34	+381.0	DIB23	+7.2
DIA42	+186.0	DIB32	+99.3
DIA62	+178.0	DIB33	+210.0
DIA63	+157.0	DIB51	+152.0
DIA64	+16.0	DIB52	+31.6
DIA72	+20.4	DIB53	+18.9
DIA73	-57.6	DIB62	+335.0
DIA74	+66.0	DIB63	+4.3
DIA82	+91.7	DIB64	-98.4
DIA83	+325.0	DIB71	+137.0
DIAX1	+145.0	DIB72	+23.5
DIAX2	+182.0	DIB82	+87.8
DIAX3	-110.0	DIB83	-57.0
		DIB84	+98.4
		DIB92	-27.0
		DIB93	+3.0

*Positive values represent an increase in ΔZ, and therefore a net demineralisation.

Table 5.7 **Rate of change in ΔZ for each lesion as calculated from the slope of the regression line: Group C (0.04ppm F) & Group D (0.06ppm F).**

Group C (0.04 ppm F) lesion	Re-/demineralisation rate*	Group D (0.06 ppm F) lesion	Re-/demineralisation rate*
DIC11	+244.0	DID11	+111.0
DIC12	+170.0	DID12	+141.0
DIC13	+466.0	DID21	+187.0
DIC21	+115.0	DID22	-16.4
DIC51	-151.0	DID31	+103.0
DIC52	-17.3	DID32	-137.0
DIC54	-64.3	DID33	+144.0
DIC61	+74.8	DID42	-46.8
DIC62	+19.0	DID52	+2.9
DIC63	+32.1	DID53	+8.6
DIC71	+142.0	DID54	+122.0
DIC72	-17.0	DID72	-36.3
DIC73	+152.0	DID73	-61.8
DIC74	-66.4	DID82	-12.0
DICX1	-38.7	DID83	+15.9
		DID84	-88.4
		DID92	-34.8
		DID93	-121.0
		DIDX1	-118.0
		DIDX2	-148.0

* Positive values represent an increase in ΔZ, and therefore a net demineralisation.

Table 5.8 **ΔZ Analysis of Variance: slope of the regression line for Groups A, B, C & D.**

Variable	Degrees of Freedom	Sum of Squares	Mean Square	F Statistic	p value
Factor	**3**	106819	35606	2.58	> 0.05
Error	**70**	976519	13822		
Total	**73**	1074338			

Table 5.9 **ΔZ Analysis of Variance : slope of the regression line /baseline ΔZ for Groups A, B, C & D.**

Variable	Degrees of Freedom	Sum of Squares	Mean Square	F Statistic	p value
Factor	**3**	142.8	47.6	2.83	<0.05
Error	**69**	1161.6	16.8		
Total	**72**	1304.5			

5.7.3 Results for Surface Zone (S.Z.).

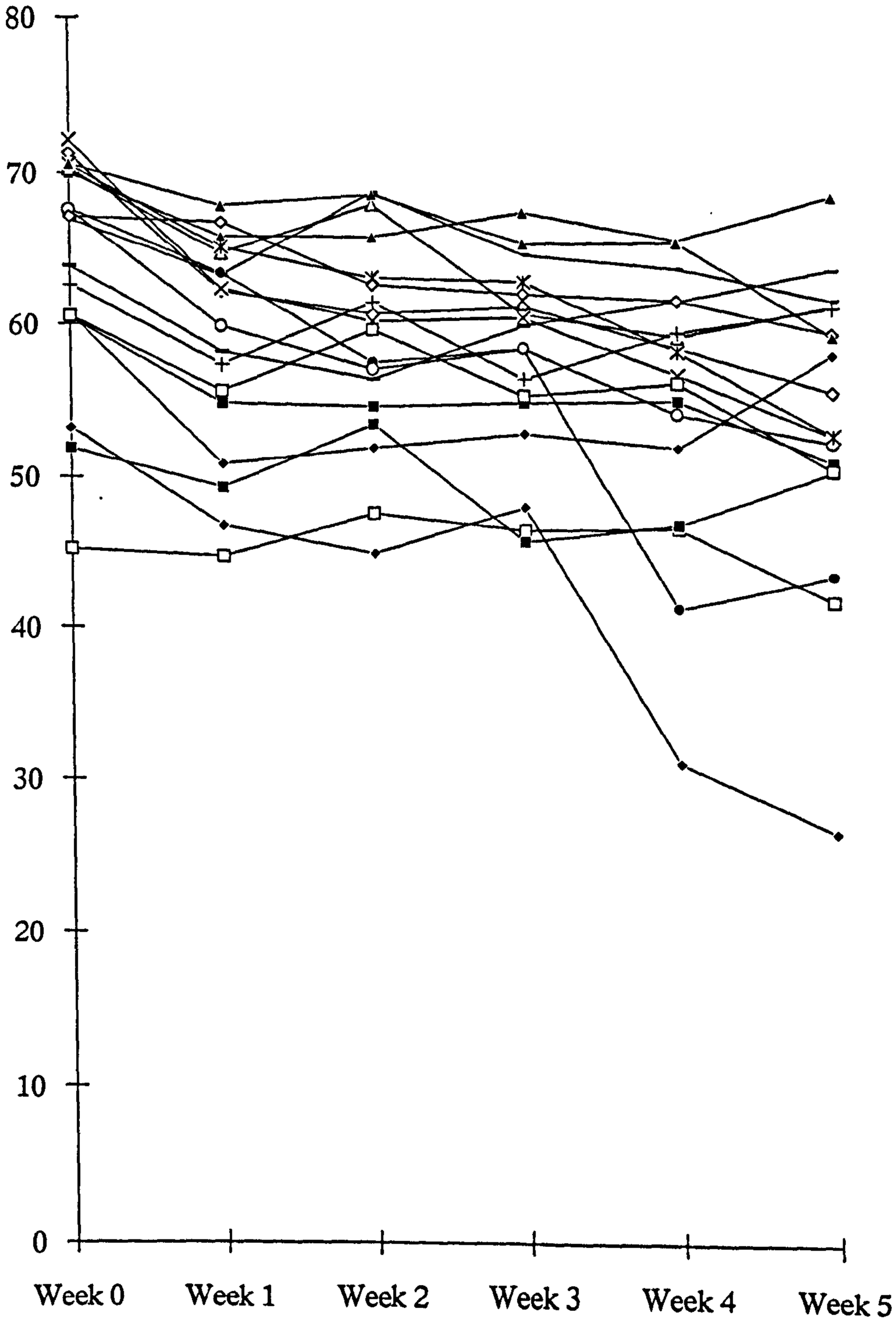
The surface zone (% vol.min.) data was subjected to exactly the same processing and analyses as ΔZ. In Figures 5.10 - 5.13, the values for the S.Z. component are plotted against time, for Groups A, B, C and D respectively. Here, a decrease in S.Z. (% vol.min.) corresponds to a reduction in mineral content, or a net demineralisation.

In Figure 5.14, the overall mean weekly change in S.Z. for each group is plotted against time. As in the corresponding figures for ΔZ and L.B. parameters, the data points lying on the y-axis represent the change in mineral content for each group over the first week of the experiment (i.e. week 1 minus week 0). These points were -5.25, -1.73, -1.86 and -1.89 for Groups A, B, C and D respectively. This initial demineralisation, most extensive in the non F Group A, was followed by a period of remineralisation in a pattern similar to ΔZ.

A visual summary of the S.Z. data is presented in Figure 5.15, as the overall mean (S.E.) change in S.Z. over the experimental period (i.e. week 5 minus week 0). Whilst this figure is provided for illustrative purposes only, it would suggest that the change in S.Z. for Group A (non F) might be significantly different from the remaining three groups. In addition, the standard error in Groups A and C seems significantly larger than in Groups B and D.

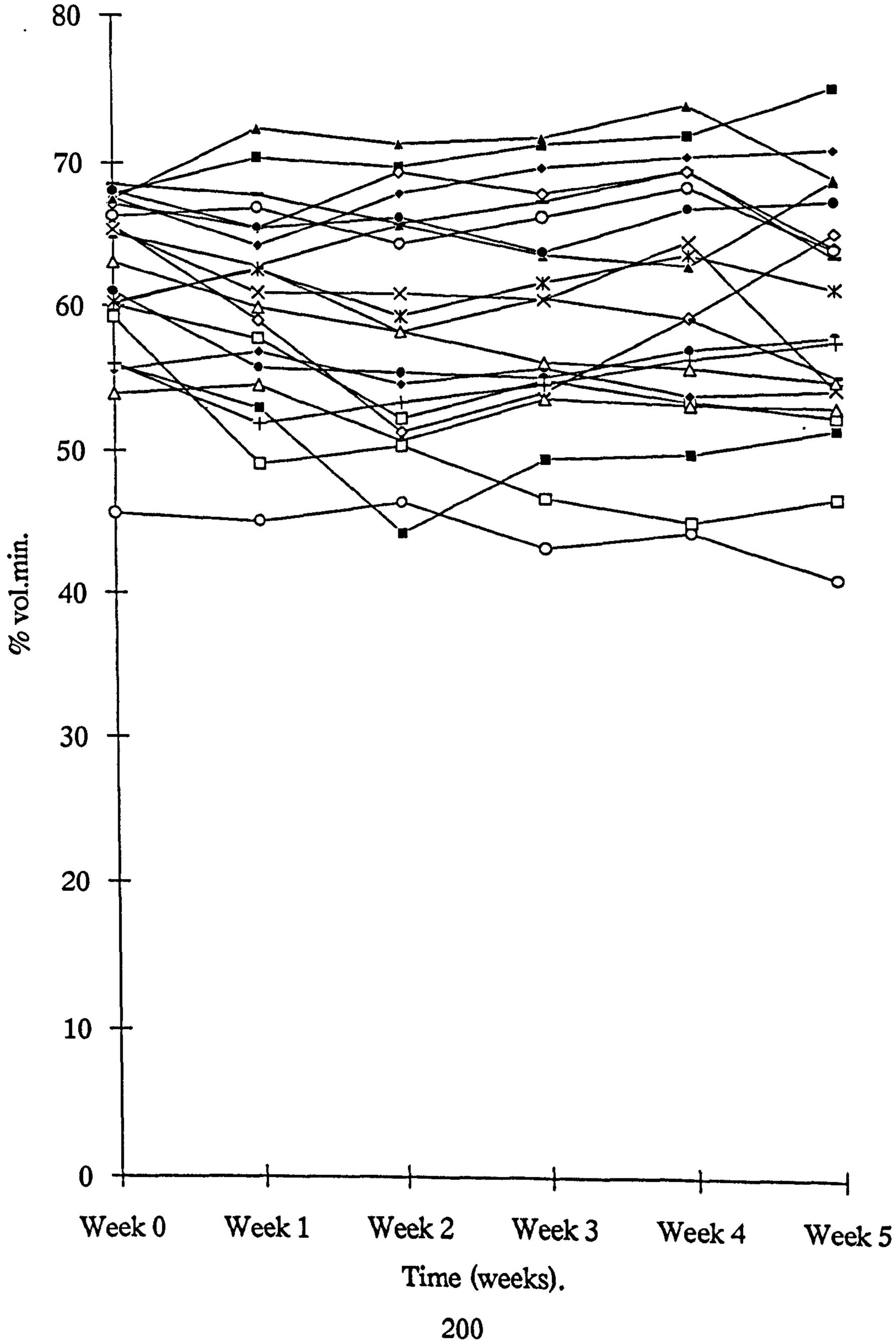
To calculate the rate of de-/remineralisation of the S.Z., a least squares fits technique was applied to generate a regression line versus time for each individual lesion, as done previously for ΔZ. The slopes of all the regression lines so generated, are equivalent to the rate of mineral change per unit time (weeks), and are presented in Tables 5.10 (Groups A & B) and 5.11 (Groups C & D). In Group A, 17 of the 18 lesions have a negative regression slope, indicating net demineralisation. For the remaining groups, 15 out of 21 lesions in Group B, six out of 15 lesions in Group C and 13 out of 20 lesions in Group D show a

Figure 5.10 Group A (non F) SZ versus time (weeks) for 18 lesions.



A decrease in %vol.min equals net demineralisation.

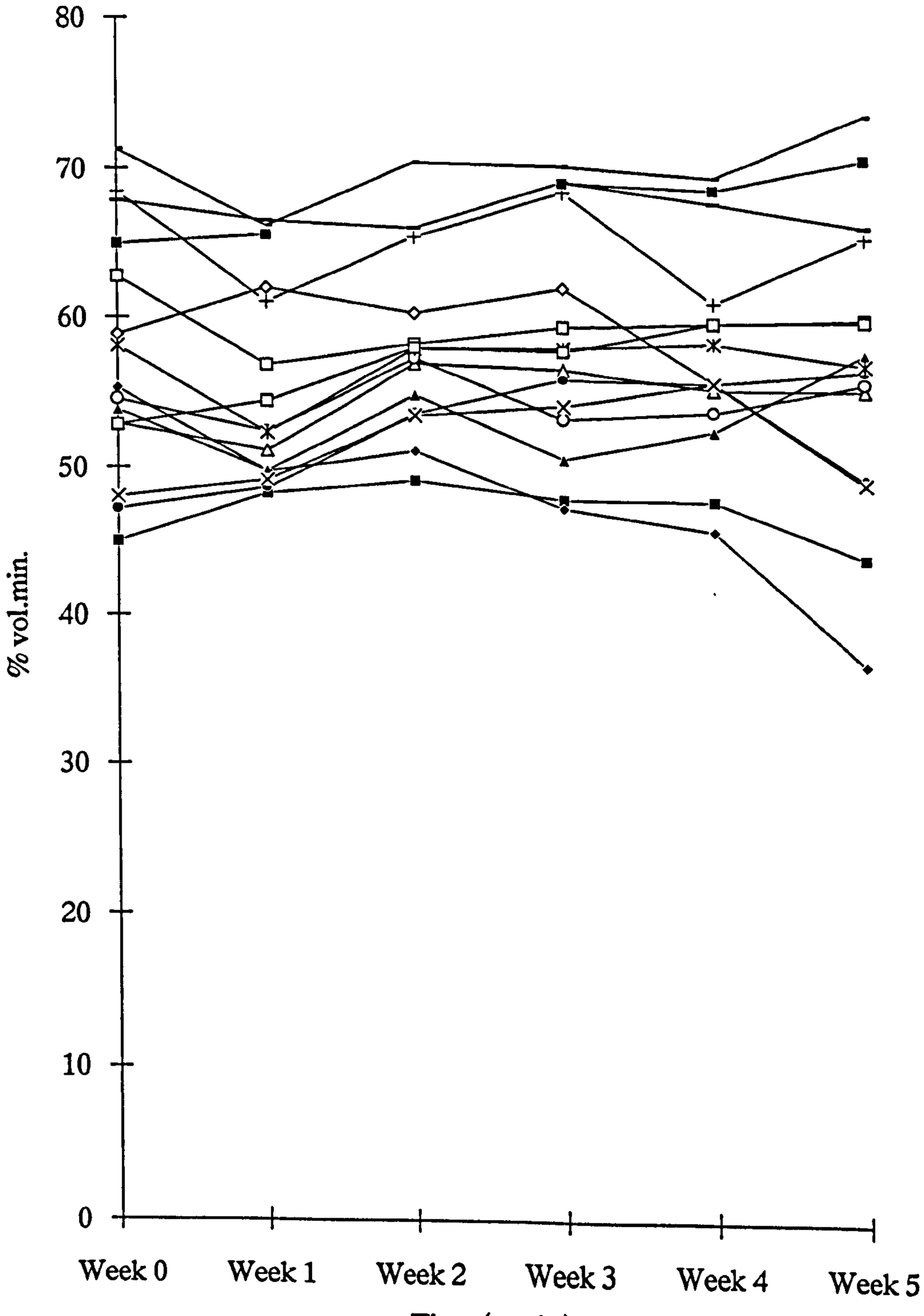
Figure 5.11 Group B (0.02 ppm F) SZ versus time (weeks) for 21 lesions.



200

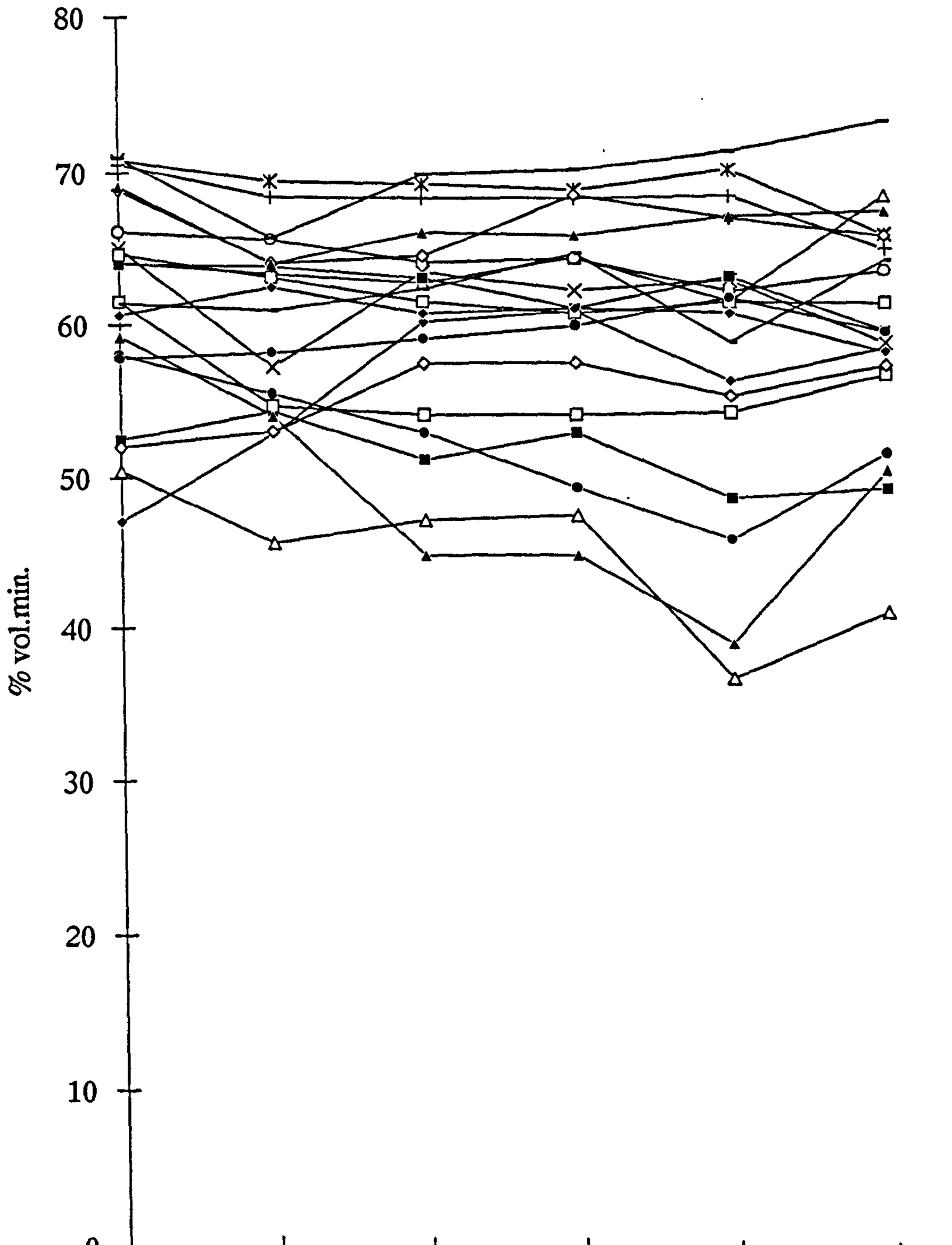
A decrease in % vol.min. equals a net demineralisation.

Figure 5.12 Group C (0.04 ppm F) SZ versus time (weeks) for 15 lesions.



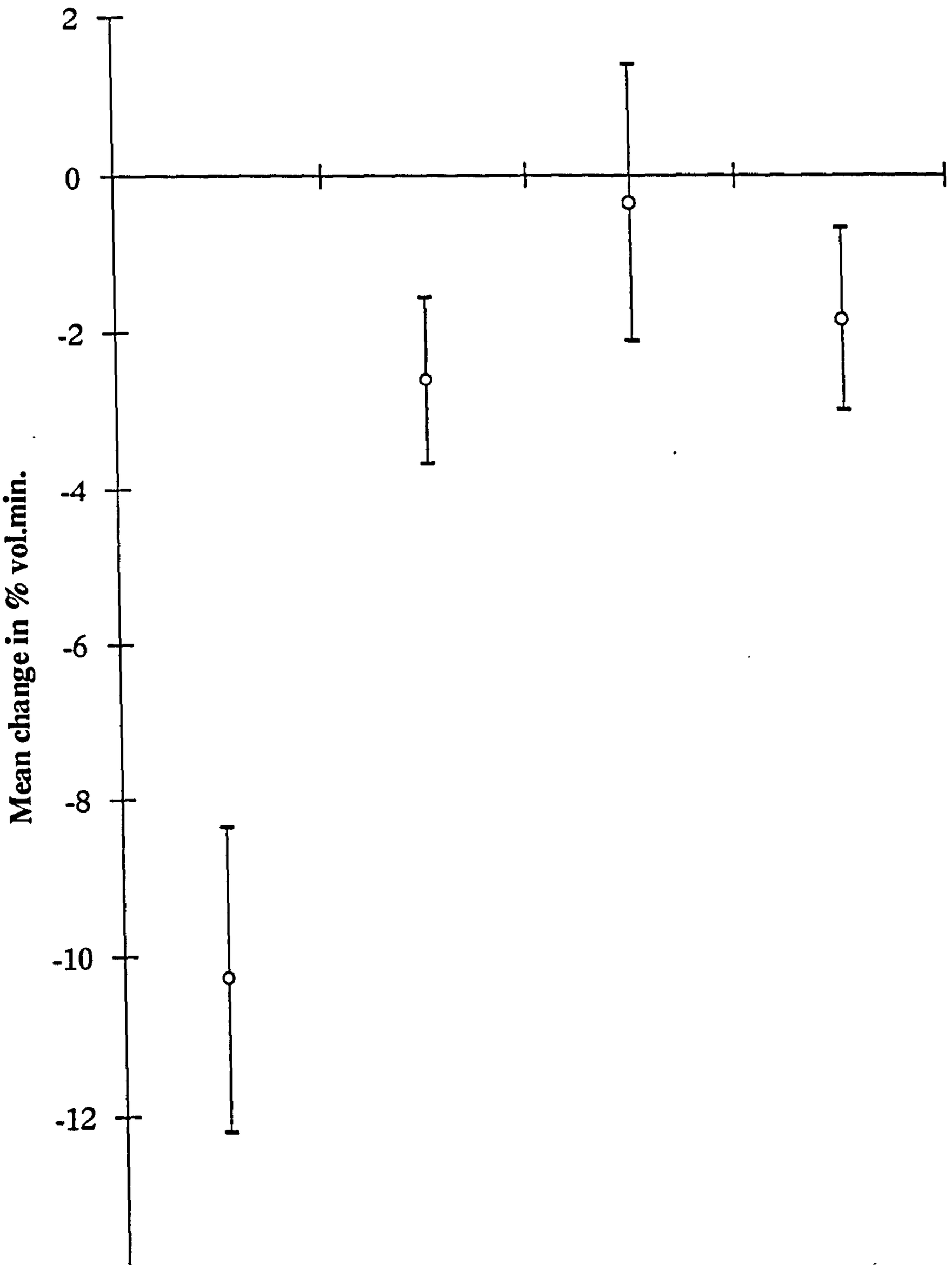
A decrease in % vol.min. equals a net demineralisation.

Figure 5.13 Group D (0.06 ppm F) SZ versus time (weeks) for 20 lesions.



A decrease in % vol.min equals a net demineralisation.

Figure 5.15 Mean (S.E.) for SZ (week 5 minus week 0) for Groups A, B, C & D.



negative regression line, corresponding to net demineralisation.

As for ΔZ, an ANOVA was conducted to determine whether there were significant differences between the re-/demineralisation rates of the S.Z. for the four groups. The results of this ANOVA are given in Table 5.12, and indicate a highly significant difference between the groups ($p < 0.001$). In addition, the individual slopes of the regression lines were divided by the initial S.Z. value for each lesion, and a second ANOVA conducted on these data. The results of this subsequent ANOVA are presented in Table 5.13, and show a very similar, highly significant difference ($p < 0.001$).

Individual comparisons of each group with the other three were then undertaken using a two-sample T-test with 99.17 % confidence intervals, to establish where these significant differences lay. The results of these analyses are given in Tables 5.18 and 5.19, and are discussed in Section 5.7.5 (Summary Statistics).

Table 5.10 **Rate of change in SZ for each lesion as calculated from the slope of the regression line: Group A (non F) & Group B (0.02ppm F).**

Group A (non F) lesion	Re-/demineralisation rate*	Group B (0.02 ppm F) lesion	Re-/demineralisation rate*
DIA13	-1.350	DIB12	-0.780
DIA14	-0.384	DIB13	-1.410
DIA31	-0.280	DIB14	-0.477
DIA32	-2.560	DIB21	-0.790
DIA33	-0.212	DIB22	0.453
DIA34	-2.000	DIB23	-0.195
DIA42	-5.320	DIB32	-0.368
DIA62	-2.660	DIB33	-0.821
DIA63	-3.270	DIB51	-1.300
DIA64	-3.150	DIB52	0.277
DIA72	-0.188	DIB53	0.617
DIA73	-0.860	DIB62	-0.967
DIA74	0.319	DIB63	-0.513
DIA82	-0.667	DIB64	1.180
DIA83	-1.530	DIB71	-2.260
DIAX1	-5.080	DIB72	1.030
DIAX2	-1.590	DIB82	-0.158
DIAX3	-1.940	DIB83	0.333
		DIB84	-1.610
		DIB92	-0.079
		DIB93	-0.173

*Positive values represent an increase in SZ mineral content i.e. a net remineralisation.

Table 5.11 **Rate of change in SZ for each lesion as calculated from the slope of the regression line: Group C (0.04ppm F) & Group D (0.06ppm F).**

Group C (0.04 ppm F) lesion	Re-/demineralisation rate*	Group D (0.06 ppm F) lesion	Re-/demineralisation rate*
DIC11	-0.213	DID11	-0.857
DIC12	-0.126	DID12	-0.704
DIC13	-3.100	DID21	1.950
DIC21	-1.860	DID22	0.977
DIC51	0.647	DID31	-2.500
DIC52	0.661	DID32	0.520
DIC54	2.000	DID33	-1.810
DIC61	0.145	DID42	-0.635
DIC62	0.723	DID52	-0.418
DIC63	0.343	DID53	-0.638
DIC71	-0.346	DID54	-0.780
DIC72	0.616	DID72	0.307
DIC73	-0.061	DID73	0.839
DIC74	2.490	DID82	-0.728
DICX1	1.430	DID83	-0.598
		DID84	-0.467
		DID92	-0.047
		DID93	0.040
		DIDX1	-2.050
		DIDX2	0.589

* Positive values represent an increase in SZ mineral content i.e. a net remineralisation.

Table 5.12 **SZ Analysis of Variance: slope of the regression line for Groups A, B, C & D.**

Variable	Degrees of Freedom	Sum of Squares	Mean Square	F Statistic	p value
Factor	**3**	39.11	13.04	8.45	<0.001
Error	**70**	108.06	1.54		
Total	**73**	147.17			

Table 5.13 **SZ Analysis of Variance : slope of the regression line /baseline SZ for Groups A, B, C & D.**

Variable	Degrees of Freedom	Sum of Squares	Mean Square	F Statistic	p value
Factor	**3**	95.84	31.95	7.11	<0.001
Error	**70**	314.34	4.49		
Total	**73**	410.18			

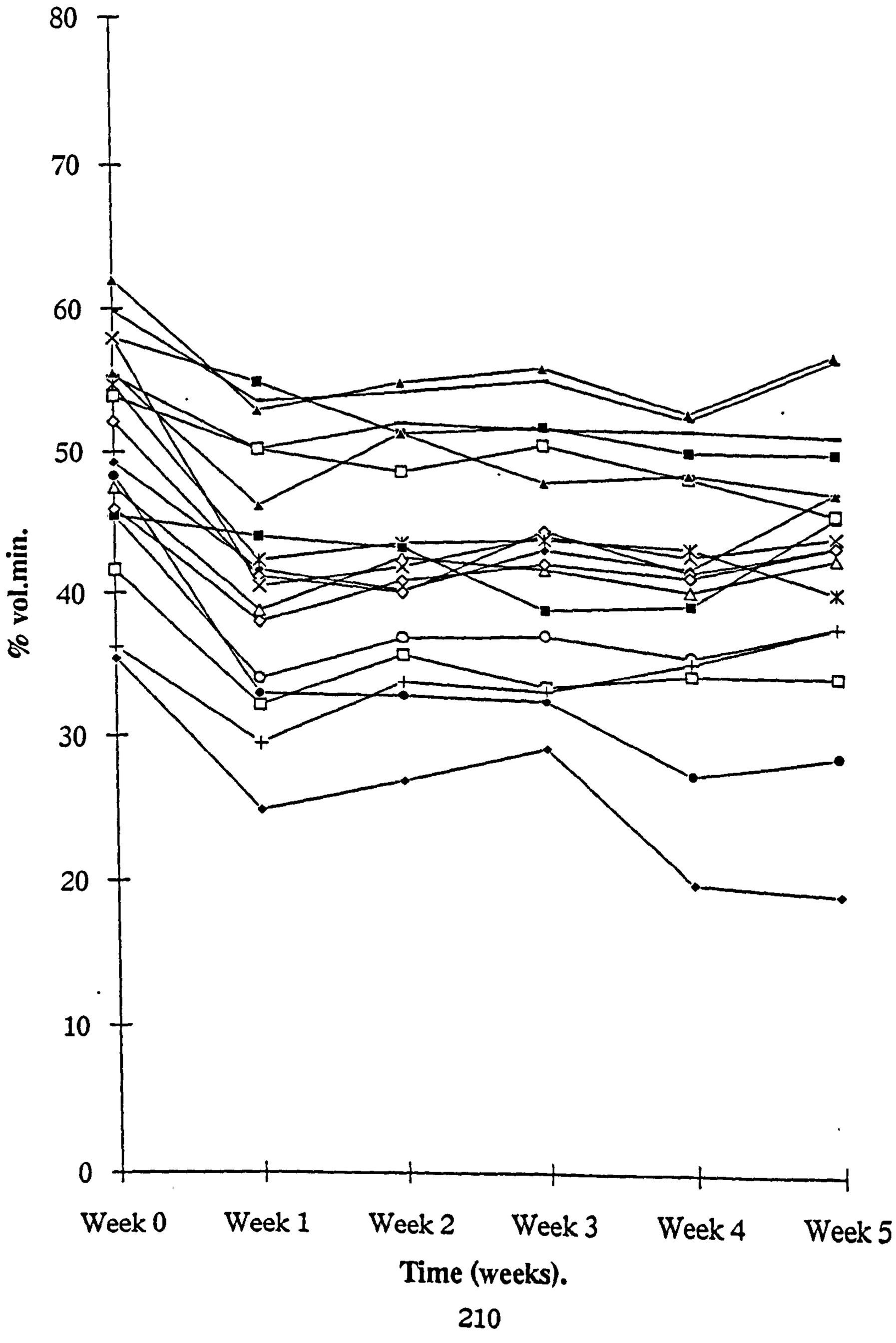
5.7.4 Results for Lesion Body (L.B.).

The processing and analyses of the data relating to the lesion body parameter (% vol.min.) was identical to that undertaken for the ΔZ and S.Z. In Figures 5.16 - 5.19, the values for L.B. mineral content are plotted against time for Groups A, B, C and D respectively. As expected, the % vol.min. content for the L.B. is significantly less than for the S.Z. For ease of comparison, the axes on Figures 5.10- 5.13 (S.Z.) and 5.16 - 5.19 (L.B.) are identical.

In Figure 5.20, the overall mean weekly change in L.B. for each group is plotted against time. As before, the data points lying on the y-axis represent the change in mineral content for each group over the first week of the experiment (i.e. week 1 minus week 0). The values for this first week were: -8.69, -5.44, -7.89 and -3.32 for Groups A, B, C and D respectively. Once again, following an initial demineralisation there appears to be a period of recovery during the second week, when all four groups remineralise the L.B. to various degrees.

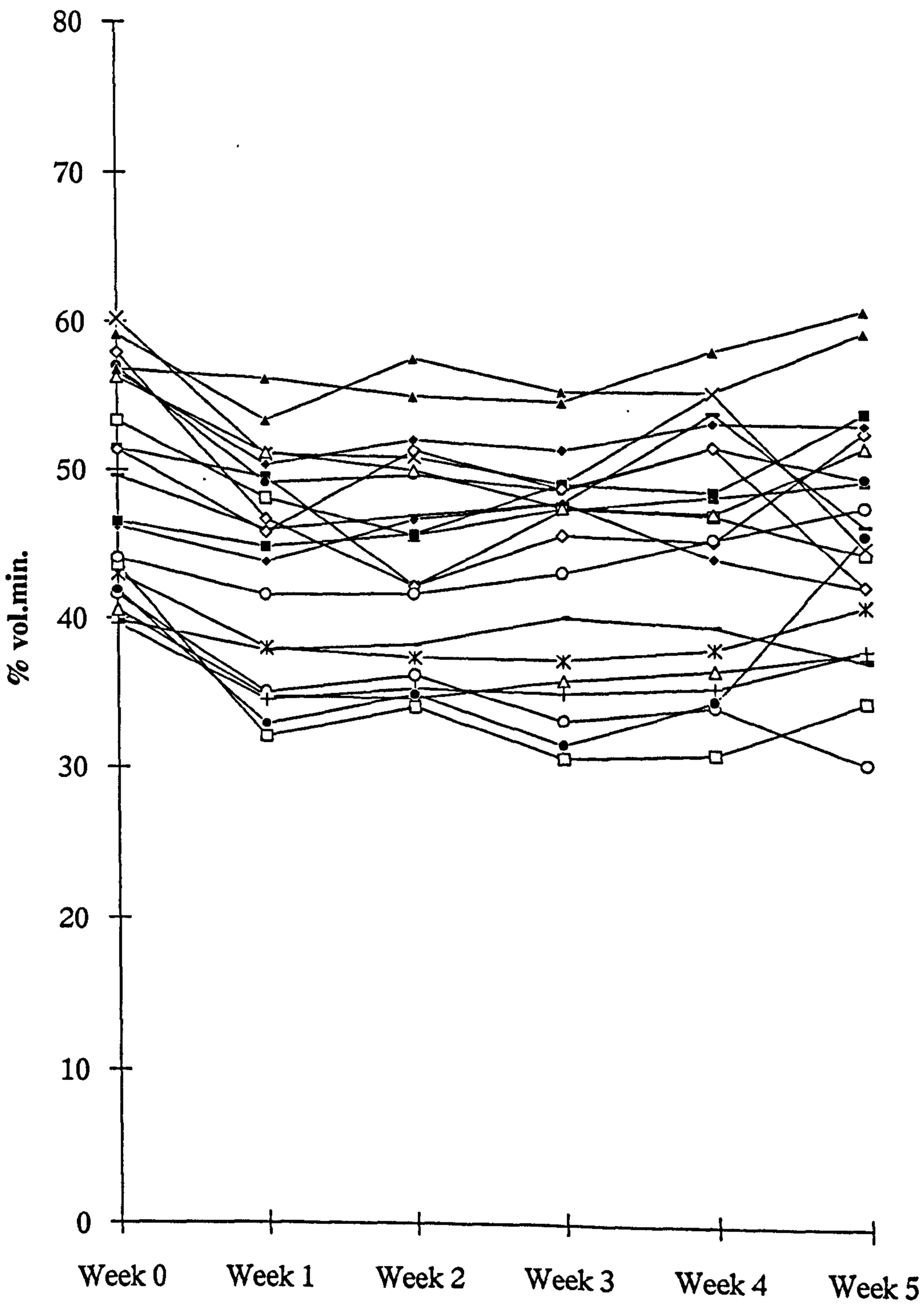
The overall mean (S.E.) change in the L.B. mineral over the experimental period is given in Figure 5.21 (week 5 minus week 0). All four groups show mean net demineralisation over the five week period, although Group D is close to zero demineralisation. Once again, Group C appears to be out of sequence with the others, although this figure is provided for illustrative purposes and should not be over-interpreted. All subsequent analyses of L.B. data are carried out on the slopes of the regression line through all the timepoints, and not simply on the difference between the first and last data points.

Figure 5.16 Group A (non-F) LB versus time (weeks) for 18 lesions.



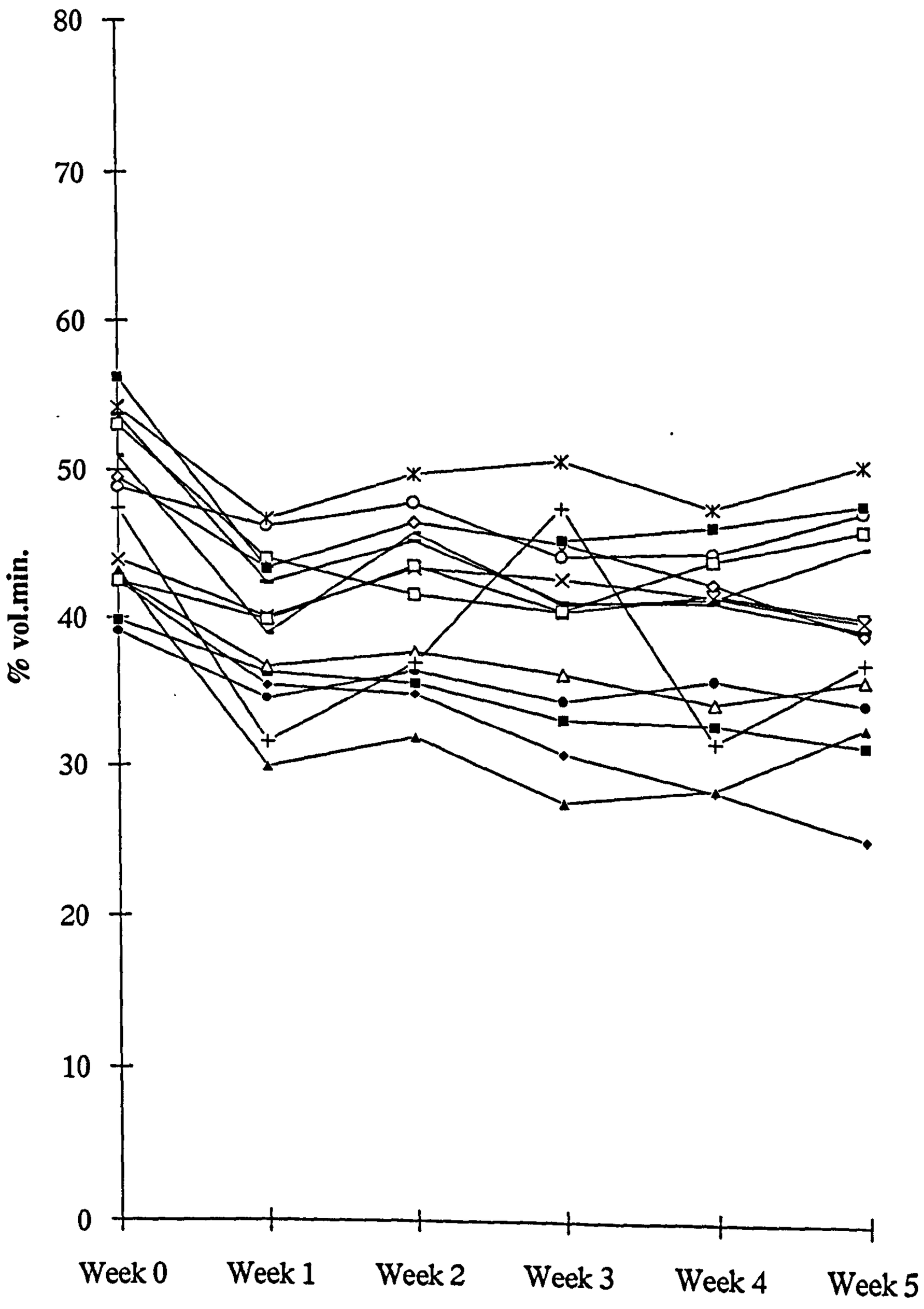
decrease in % vol.min. equals a net demineralisation.

Figure 5.17 Group B (0.02 ppm F) LB versus time (weeks) for 21 lesions.



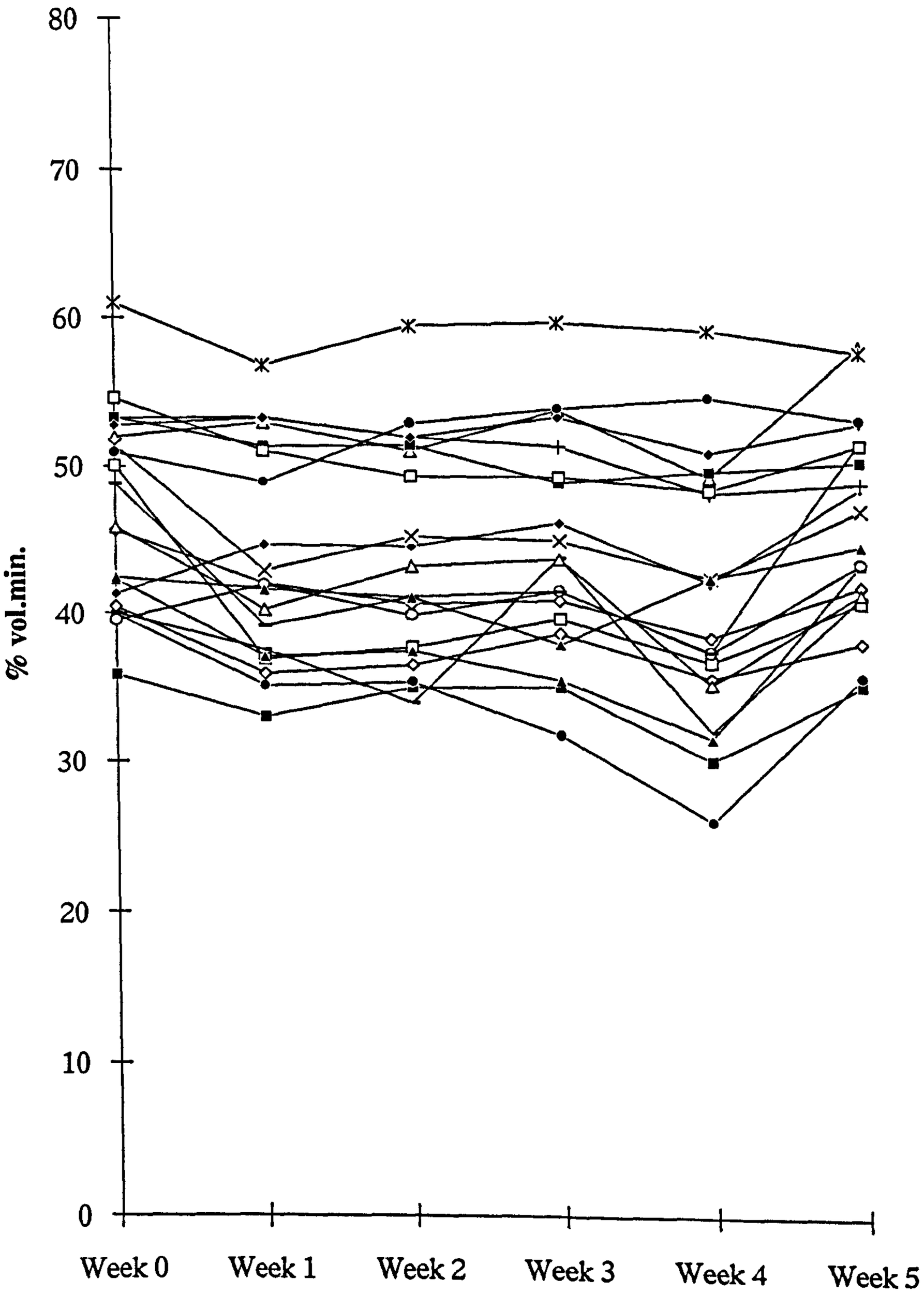
A decrease in % vol.min. equals a net demineralisation.

Figure 5.18 Group C (0.04 ppm F) LB versus time (weeks) for 15 lesions.



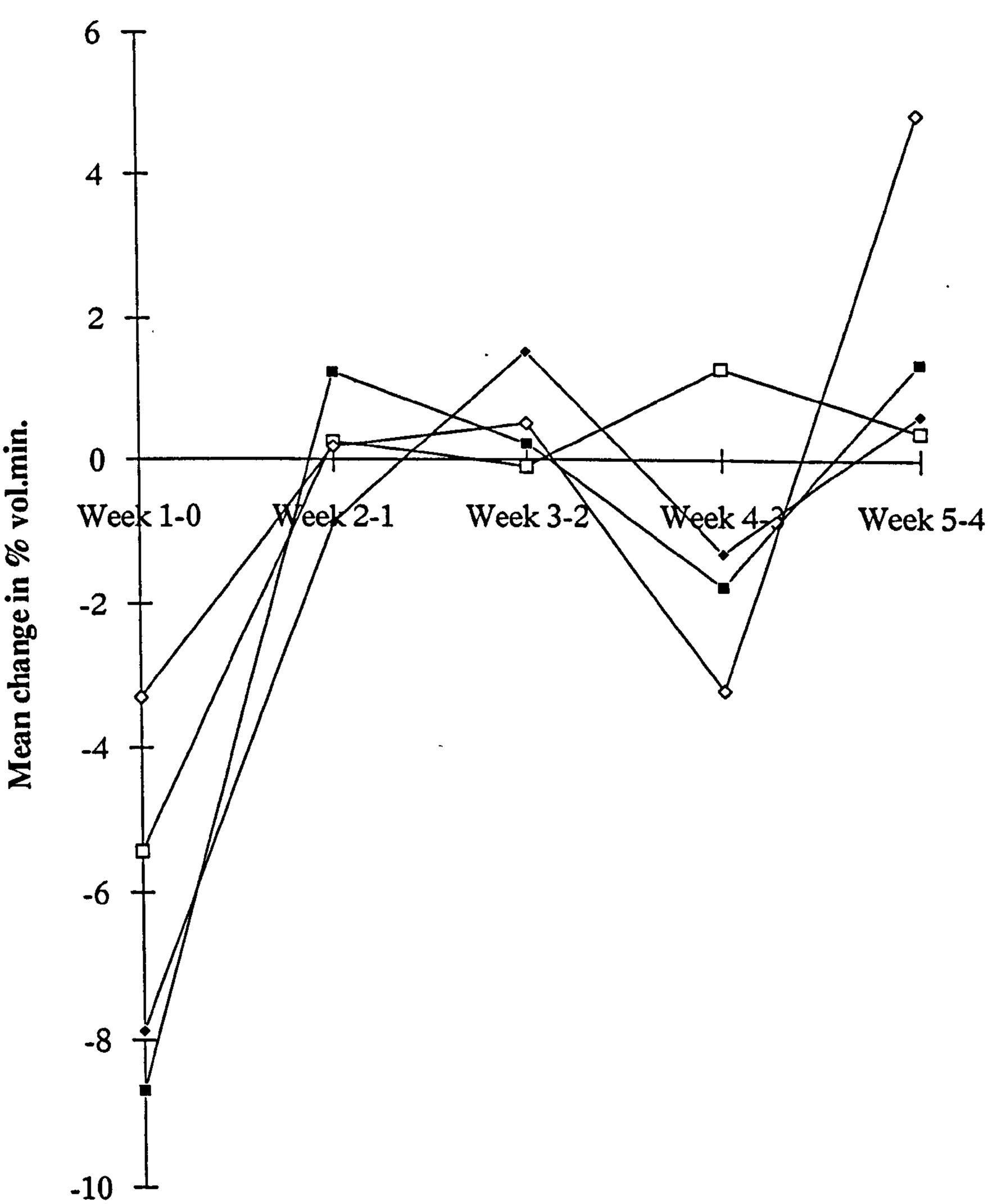
A decrease in % vol.min. equals a net demineralisation.

Figure 5.19 Group D (0.06 ppm F) LB versus time (weeks) for 20 lesions.



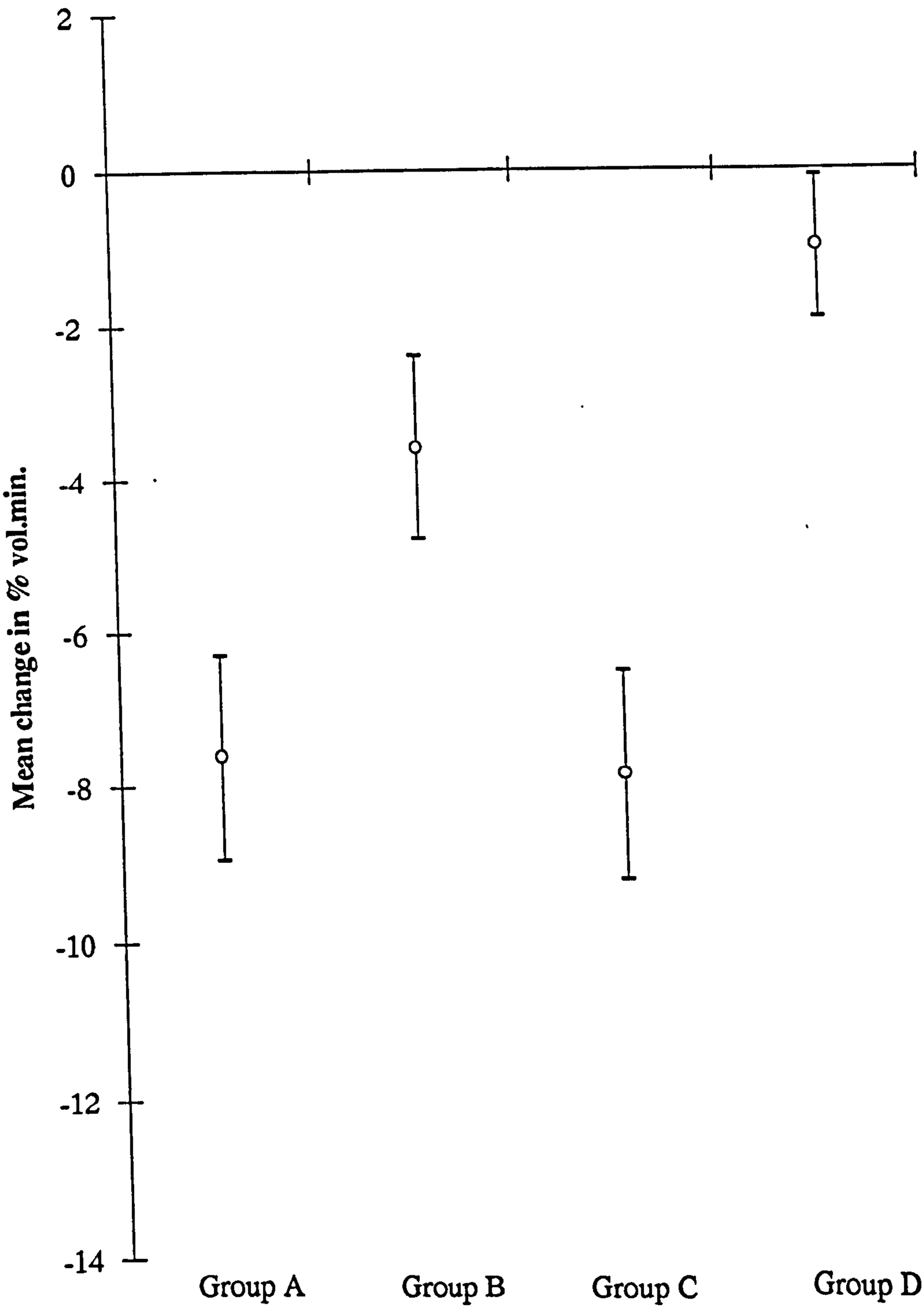
A decrease in % vol.min. equals a net demineralisation.

Figure 5.20 Mean change in LB with time (weeks) for all lesions in each of Groups A, B, C & D.



A decrease in % vol.min equals a net demineralisation.

Figure 5.21 Mean (S.E.) for LB (week 5 minus week 0) for Groups A, B, C, & D.



For each individual lesion, a regression line was drawn using a least squares fits technique. The slopes of the regression lines are listed in Table 5.14 (Groups A & B) and Table 5.15 (Groups C & D). As with the SZ parameter, 17 of 18 lesions in Group A had a negative slope to the regression line, indicating that all but one lesion underwent a net loss of mineral from the lesion body. Furthermore, 15 of 20 lesions in Group B, 14 of 15 lesions in Group C and 14 of 20 lesions in Group D also had negative regression line slopes, and therefore net demineralisation rates.

Interestingly, far more lesions in Group C underwent net demineralisation from the LB than from the SZ (14 lesions as opposed to six). In fact, a total of eight lesions out of 15 in this group showed demineralisation from the LB, but remineralisation of the SZ.

The results for the ANOVA on these data are presented in Tables 5.16 (slope of the regression line) and 5.17 (slope of the regression line /baseline LB). In both instances, there are significant differences between the groups ($p < 0.01$). The p values for both ANOVA's are very similar, 0.007 and 0.006, and it appears, therefore, that including the baseline LB value in the analysis has only a very small impact on the results.

To identify where the significant differences between the groups lay, individual comparisons of each group with the others was undertaken, using a two sample T-test with 99.17% C.I.'s. The results of the individual comparisons for all three parameters (ΔZ, SZ and LB) and the results of an Analysis of Co-Variance (ANCOVA) are provided in the next section.

Table 5.14 **Rate of change in LB for each lesion as calculated from the slope of the regression line: Group A (non F) & Group B (0.02ppm F).**

Group A (non F) lesion	Re-/demineralisation rate*	Group B (0.02 ppm F) lesion	Re-/demineralisation rate*
DIA13	-1.760	DIB12	-0.287
DIA14	-0.972	DIB13	-1.330
DIA31	-0.239	DIB14	-0.541
DIA32	-1.140	DIB21	-0.790
DIA33	-0.773	DIB22	0.120
DIA34	-0.654	DIB23	-0.199
DIA42	-3.340	DIB32	-0.895
DIA62	-0.983	DIB33	-1.780
DIA63	-1.780	DIB51	-1.880
DIA64	-2.030	DIB52	-0.310
DIA72	0.647	DIB53	-0.175
DIA73	-0.621	DIB62	-0.197
DIA74	-0.524	DIB63	0.223
DIA82	-0.579	DIB64	1.460
DIA83	-1.260	DIB71	-2.420
DIAX1	-2.730	DIB72	-0.291
DIAX2	-0.116	DIB82	-0.901
DIAX3	-1.140	DIB83	0.696
		DIB84	-1.100
		DIB92	0.580
		DIB93	0.869

*Positive values represent an increase in LB mineral content i.e. a net remineralisation.

Table 5.15 **Rate of change in LB for each lesion as calculated from the slope of the regression line: Group C (0.04ppm F) & Group D (0.06ppm F).**

Group C (0.04 ppm F) lesion	Re-/demineralisation rate*	Group D (0.06 ppm F) lesion	Re-/demineralisation rate*
DIC11	-1.590	DID11	-0.381
DIC12	-2.110	DID12	-1.320
DIC13	-3.210	DID21	0.821
DIC21	-1.640	DID22	-0.871
DIC51	-1.770	DID31	-0.400
DIC52	-1.220	DID32	0.588
DIC54	-0.651	DID33	-1.510
DIC61	-0.503	DID42	0.172
DIC62	-0.499	DID52	-0.174
DIC63	-0.475	DID53	-0.308
DIC71	-1.190	DID54	-1.130
DIC72	-0.830	DID72	-0.070
DIC73	-2.310	DID73	0.250
DIC74	-0.240	DID82	-0.688
DICX1	0.731	DID83	-0.726
		DID84	-0.176
		DID92	-0.335
		DID93	0.301
		DIDX1	-1.090
		DIDX2	0.808

* Positive values represent an increase in LB mineral content i.e. a net remineralisation.

Table 5.16 **LB Analysis of Variance: slope of the regression line for Groups A, B, C & D.**

Variable	Degrees of Freedom	Sum of Squares	Mean Square	F Statistic	p value
Factor	**3**	10.774	3.591	4.51	<0.01
Error	**70**	55.702	0.796		
Total	**73**	66.476			

Table 5.17 **LB Analysis of Variance : slope of the regression line /baseline LB for Groups A, B, C & D.**

Variable	Degrees of Freedom	Sum of Squares	Mean Square	F Statistic	p value
Factor	**3**	49.24	16.41	4.33	<0.01
Error	**70**	265.14	3.79		
Total	**73**	314.38			

5.7.5 Summary Statistics.

In order to identify which groups were significantly different from the others, it was necessary to carry out individual comparisons between the groups, using two-sample T-tests, with the confidence value adjusted to 99.17% to compensate for the multiple comparisons (Groups A v B, A v C, A v D, B v C, B v D and C v D = six comparisons ∴ 0.05 ÷ 6 = 0.0083, and 1- 0.0083 = 0.9917 ∴ C.I. should be 99.17 instead of 95).

In Table 5.18, the results of the comparisons of the slope of regression lines is summarised. The only significant difference identified for the ΔZ parameter was between Group A (non F) and Group D (0.06 ppm F), $p < 0.01$, although the comparison of Group B with Group D produced a p value of borderline significance at 0.079.

In contrast, the comparisons of the SZ data produced highly significant differences ($p < 0.005$) between Group A (non F) and all the other fluoridated groups. These results were reflected in the highly significant p value in the SZ ANOVA (Table 5.12) . For the remaining comparisons, B v C, B v D and C v D, no differences were detected.

Finally, the comparisons of the LB data produce rather more conflicting results:- Group A was significantly different from Group B ($p < 0.05$), highly significantly different from Group D ($p < 0.01$) but was not different from Group C. Furthermore, the three remaining comparisons were as follows:- B v C, $p < 0.05$, B v D, $p > 0.05$ and C v D, $p < 0.01$. As discussed in the previous section, eight of the 15 lesions in Group C lost mineral from the lesion body, whilst gaining mineral in SZ, which may have contributed to these conflicting results.

Table 5.18 **Summary of comparisons of all four groups (ΔZ, SZ & LB): slope of the regression lines** ***(Two-sample T-test, 99.17% C.I.)***

	ΔZ				SZ				LB			
	A	B	C	D	A	B	C	D	A	B	C	D
A	-	NS	NS	**								
B	NS	-	NS	NS								
C	NS	NS	-	NS								
D	**	NS	NS	-								
A					-	***	***	***				
B					***	-	NS	NS				
C					***	NS	-	NS				
D					***	NS	NS	-				
A									-	*	NS	**
B									*	-	*	NS
C									NS	*	-	**
D									**	NS	**	-

NS = not significant (p≥0.05)
*** = 0.01< p < 0.05**
**** = 0.005 < p < 0.01**
***** = p < 0.005**

A summary of the multiple comparisons made on the slope/baseline data is provided in Table 5.19. Once again, 99.17% was set as the confidence interval. The pattern of results is very similar to that in the previous table, although introducing the baseline mineral content of each of the parameters into the analysis has had the effect of reducing some of the differences between the groups. For example, the p value for the comparison of Groups A and D (ΔZ) changes from 0.008 to 0.017. Similarly, the p value for the comparison between Groups A and D (SZ) changes from 0.003 to 0.005. Overall, however, the pattern of significant versus non-significant differences remains unaltered following the introduction of baseline mineral content.

As there has been considerable interest in baseline mineral content as an influential factor in determining lesion behaviour, a final analysis of these data was undertaken. Using the baseline mineral content for each lesion as a co-variate, Analysis of Co-Variance (ANCOVA) was undertaken for each parameter (ΔZ, SZ and LB). A generalised linear model on *Minitab Release 9.0* software (*Minitab Incorporated)* was used. The results of the ANCOVA are presented in Tables 5.20, 5.21 and 5.22 for ΔZ, SZ and LB respectively.

As with the earlier ANOVA's, statistically significant differences between the groups for SZ and LB were identified, with ΔZ comparisons producing a borderline p value of 0.064. However, the inclusion of the baseline mineral content as a co-variate produced p values of 0.86 (ΔZ), 0.65 (SZ) and 0.49 (LB), and was therefore not a significant factor in determining how the lesions in this *in vitro* experiment behaved.

Table 5.19 **Summary of comparisons of all four groups (ΔZ, SZ & LB): slope of the regression lines/baseline value *(Two-sample T-test, 99.17% C.I.)*.**

	ΔZ				SZ				LB			
	A	B	C	D	A	B	C	D	A	B	C	D
A	-	NS	NS	*								
B	NS	-	NS	NS								
C	NS	NS	-	NS								
D	*	NS	NS	-								
A					-	***	***	**				
B					***	-	NS	NS				
C					***	NS	-	NS				
D					**	NS	NS	-				
A									-	*	NS	*
B									*	-	*	NS
C									NS	*	-	**
D									*	NS	**	-

NS = not significant ($p \geq 0.05$)

* = $0.01 < p < 0.05$

** = $0.005 < p < 0.01$

*** = $p < 0.005$

Table 5.20 **ΔZ Analysis of Co-Variance on slope of the regression line (i.e. rate of change in ΔZ) with baseline ΔZ as a co-variate for Groups A, B, C & D.**

Variable	Degrees of Freedom	Sequential Sum of Squares	Adjusted Sum of Squares	Adjusted Mean Square	F Statistic	p value
Group	**3**	106819	106603	35534	2.54	0.064
Baseline ΔZ	**1**	428	428	428	0.03	0.862
Error	**69**	967091	967091	14016		
Total	**73**	1074338				

Table 5.21 **SZ Analysis of Co-Variance on slope of the regression line (i.e. rate of change in SZ) with baseline SZ as a co-variate for Groups A, B, C & D.**

Variable	Degrees of Freedom	Sequential Sum of Squares	Adjusted Sum of Squares	Adjusted Mean Square	F Statistic	p value
Group	**3**	39.113	34.708	11.569	7.41	0.000
Baseline SZ	**1**	0.323	0.323	0.323	0.21	0.651
Error	**69**	107.734	107.734	1.561		
Total	**73**	147.169				

Table 5.22 **LB Analysis of Co-Variance on slope of the regression line (i.e. rate of change in LB) with baseline LB as a co-variate for Groups A, B, C & D.**

Variable	Degrees of Freedom	Sequential Sum of Squares	Adjusted Sum of Squares	Adjusted Mean Square	F Statistic	p value
Group	**3**	10.5611	10.5611	3.5204	4.39	0.007
Baseline LB	**1**	0.5924	0.3795	0.3795	0.47	0.494
Error	**69**	55.3221	55.3221	0.8018		
Total	**73**	66.4756				

5.8 Discussion.

Several studies support the hypothesis that daily use of fluoride products is required to elevate salivary and plaque fluoride concentrations and to provide fluoride at the time of acid challenge and subsequent remineralisation (Koulourides, Cueto & Pigman,1961; Feagin *et al.*1971; Moreno & Zahradnik,1979; Joyston-Bechal & Kidd,1982; ten Cate & Duijsters,1983; Featherstone *et al.*1986). In addition, published data (Amjad & Nancollas,1979) has demonstrated that fluoride concentrations as low as 0.1 ppm may be sufficient to enhance apatite crystal growth. If the presence of fluoride in concentrations < 0.1ppm is potentially therapeutic, it is important that any new anti-caries product continues to deliver fluoride in this concentration range for prolonged periods. With the introduction of lower fluoride dentifrices, largely intended for use in children, it is important that the ambient levels of fluoride in the oral cavity are maintained.

This study was designed to investigate the impact of ambient fluoride levels from lower concentration dentifrices, as determined in the previous *in vivo* study (Chapter 4), on early enamel lesion mineralisation rates. The results show a significant effect of fluoride concentration on ΔZ, SZ and LB mineralisation rates when initial lesion size is taken into consideration. Increasing the fluoride concentration reduced the rate of demineralisation and, for the highest fluoride group (0.06 ppm F), produced a net remineralisation for ΔZ.

Whilst initial lesion size had a marginal effect on the result of ΔZ, it had less overall impact than had been anticipated. It may be that, in the *in vitro* situation, and with fluoride in both the re- and demineralising solutions, initial lesion size is a less significant factor than in the *in situ* situation.

Overall, these results are in broad agreement with an *in vitro* study undertaken (Damato,1990) to investigate the difference in response of solution-prepared and gelatin-prepared lesions to a similar range of sub-ppm fluoride concentrations, although in this

previous study, the fluoride was present only in the remineralising solutions. This earlier study also showed a significant effect of fluoride on ΔZ and SZ mineralisation rates, although the results for the LB parameter were not significant.

Extrapolation of these results to the *in vivo* situation is not straightforward, as the intra-oral environment produces a very complex pattern of fluoride distribution and clearance (Weatherell *et al.*1984; Dawes & Weatherell,1990; Jacobson, Stephen & Strang,1992). Salivary fluoride is elevated after topical application and the degree of fluoride elevation during the post-application period depends on many factors (see Chapter 6), including the fluoride concentration of the fluoride source, endogenous sources such as microbial and epithelial cells, and on the rate of fluoride clearance (Ekstrand, Lagerlöf & Oliveby,1986) from the oral cavity. Dawes (1983) showed that the rate of oral clearance of a solute depends most importantly on the volume of saliva in the mouth before and after swallowing, and on the salivary flow rate. Such findings , together with the complexity of fluoride clearance at different sites in the mouth (Weatherell *et al.*1988), demonstrate that developing fluoride regimens to deliver maximum therapeutic benefit is an exacting task. Nonetheless, since it is not possible to establish clinically exactly when caries occurs, it is logical to continue to recommend frequent exposure of enamel to agents containing low concentrations of fluoride (Joyston-Bechal & Kidd,1982).

Finally, the results of this study suggest that low levels of fluoride (< 0.1 ppm) in solution can have a significant impact on artificial caries lesion mineralisation rates, particularly on the surface of the lesion. The fluoride concentrations tested here correspond to the ambient levels found *in vivo* in the preceding experiment (Chapter 4), when subjects used dentifrices containing 1500, 1000, 500 and 0.22 ppm fluoride. It would seem to be important that the efficacy of the new lower fluoride-containing dentifrices is investigated in full, double-blind clinical trials as soon as possible.

Chapter 6. Investigations into Fluoride Clearance.

6.1 Introduction: Peak versus Baseline.

Whilst the efficacy of fluoride in reducing the incidence and rate of progression of dental caries is widely accepted, the details of the mechanism of action are still debated. For example, as discussed in the previous chapter, the actual concentration of fluoride required to completely inhibit demineralisation is not known. Similarly, the relative benefit of the initial high, but short-lived concentration of fluoride found intra-orally after topical application, versus the prolonged period of gradually reducing fluoride levels which follows, is not yet established. This is an important factor in developing new efficacious agents, as the high or peak concentrations are those responsible for the deleterious effects of fluoride therapy, such as gastric erosion and fluorosis, which could be eliminated if prolonged, lower levels were deemed equally effective. Furthermore, while the patterns of fluoride clearance from plasma and saliva are well characterised and are demonstrated and discussed in this chapter, the potential role of the oral soft tissues in intra-oral fluoride clearance required investigation.

6.2 Plasma Fluoride Clearance.

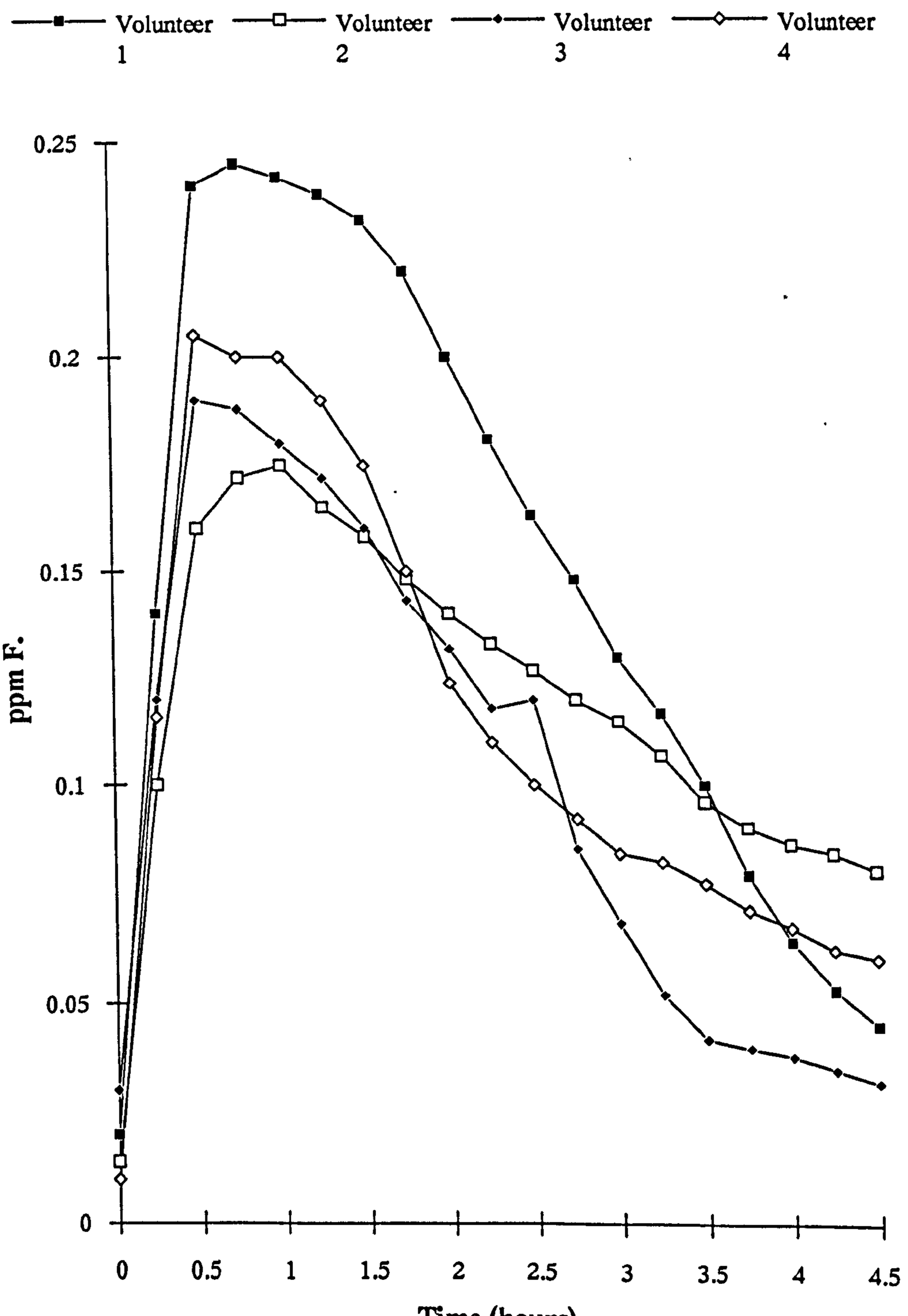
The pharmacokinetics and metabolism of fluoride are detailed in Section 1.3.8, and the relationship between plasma fluoride and fluorosis is discussed in Section 4.1.2. It is only necessary here, therefore, to re-iterate that a number of studies (Ekstrand,1978; Angmar-Månsson & Whitford,1982) have concluded that small but recurring elevations in plasma fluoride to around 0.05-0.20 ppm (3-9 μM), during the period of enamel development, are most commonly associated with cases of fluorosis. In addition, higher levels of fluorosis have been observed in young children ingesting fluoride tablets as a single daily dose when

compared to a population ingesting the same level of fluoride in fluoridated water.

To demonstrate the clearance pattern of fluoride from plasma, four adult volunteers were recruited, and instructed to use a non-fluoridated dentifrice for the 24 hours preceding the experiment. A baseline plasma sample was collected from each volunteer, using the micromethod described in Chapter 4. All samples were stored at -16°C until required for analysis. Each volunteer was then given a 3 mg oral dose of sodium fluoride (equivalent to 1.5 mg F), which consisted of three x 1 mg sodium fluoride tablets in a soluble gelatin capsule. Plasma samples were then collected at 15 minute intervals over the next four and a half hours. All fluoride measurements were conducted in accordance with the techniques described in Appendix 3.4.

The clearance pattern for all four volunteers is given in Figure 6.1. It can be seen that there is a very rapid rise in plasma fluoride levels for all volunteers, with peak values occurring in the first 30-45 minutes. These peak values ranged from 0.175 - 0.245 ppm, and all volunteers had plasma fluoride levels greater than the "threshold" levels discussed above for three and a half to four hours. Following the peak concentration, there is a steady decline in plasma fluoride, with a half-life varying between two and four hours. This relatively short half-life (normally quoted as four to ten hours) may be an indication that the time period of sampling should have been more prolonged, to ensure the slope of the elimination curve was not artificially steep. In addition, all the volunteers had elevated plasma fluoride levels at the final sampling (four and a half hours), indicating that a further period of elimination had still to occur. In general, the elimination of fluoride from plasma is *via* renal excretion (Carlson, Armstrong & Singer,1960; Whitford, Pashley & Stringer,1976; Oliveby *et al.*1989a; Whitford,1994)) and by skeletal sequestration, which takes up about one quarter to one half of the dose absorbed (Largent & Heyworth,1949; Hodge,1956).

Figure 6.1 Plasma fluoride clearance following 3 mg oral dose of NaF.



Overall, however, this study demonstrates the typical pattern of plasma fluoride clearance, with rapid absorption from the gastro-intestinal tract, and gradual elimination over the subsequent hours. The absolute values recorded here are not of significance, as the dose of 3 mg NaF was adopted to illustrate the clearance pattern, and was not intended to represent a therapeutic regimen.

6.3 Salivary Fluoride Clearance.

There are two major sub-divisions of salivary clearance studies in the published literature. These are firstly, studies monitoring the concentration of fluoride in duct saliva, usually from the parotid gland, as a measure of systemic fluoride uptake and re-circulation (Yao & Gron,1970; Ekstrand,1979; Oliveby *et al.*1989a) and secondly, studies monitoring the clearance of fluoride from whole, mixed saliva as a measure of the bio-availablity of fluoride from topical agents such as dentifrices and mouth-rinses (Bruun, Givskov & Thylstrup,1984; Bruun & Thylstrup,1987; Bruun, Qvist & Thylstrup,1987; Oliveby, Ekstrand & Lagerlöf,1987; Zero *et al.*1988; Duckworth & Morgan,1992; Duckworth, Morgan & Gilbert,1992; Sjögren & Birkhed,1993).

6.3.1 Duct Salivary Fluoride.

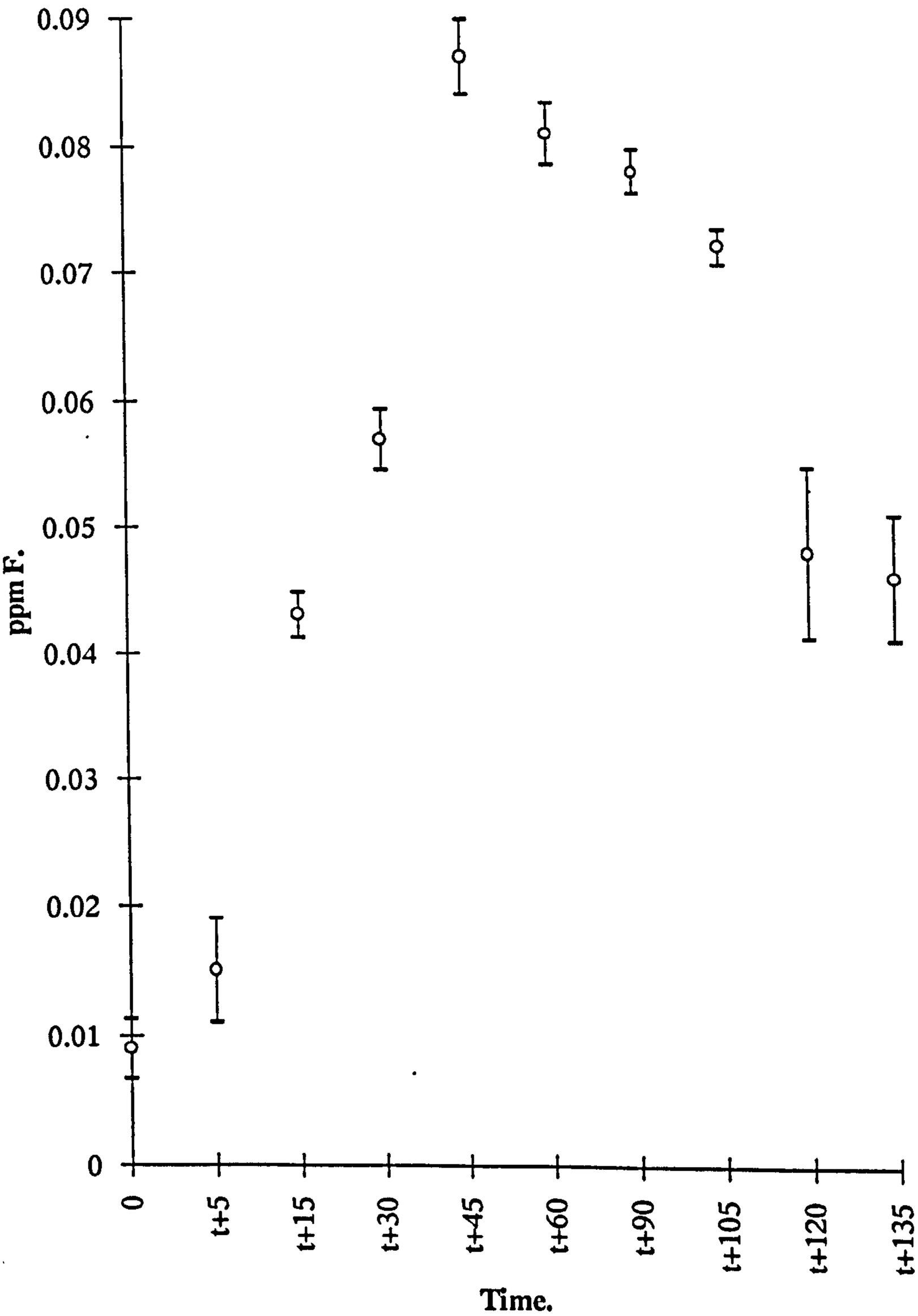
The fluoride found in duct saliva is derived from the central compartment, i.e. from plasma, and the concentrations of fluoride found in saliva, before entering the oral cavity, are closely associated with plasma levels. This relationship, usually termed the saliva/plasma ratio (S/P ratio), has been variously quoted as 0.3-0.6 (Oliveby *et al.*1989a,c), 0.64 (Ekstrand *et al.*1977) and 1.0 (Ericsson,1969). The equivalence of saliva and plasma levels reported by Ericsson has not been repeated, and an S/P ratio of 0.4-0.6 is generally accepted. There are two possible mechanisms by which this difference in concentration could occur: either a concentration gradient exists at the plasma:tissue fluid interface, whereby only a portion of the plasma fluoride is absorbed into the gland tissue, or all the plasma fluoride diffuses into

the gland tissue, but some is reabsorbed by the salivary gland ductal system immediately prior to secretion. The precise mechanism determining this S/P ratio is not yet established.

Following ingestion of an oral fluoride dose, the peak concentration in saliva tends to be slightly more delayed than that in plasma. This may be explained partly by the lag-time required for fluoride transport from the blood vessels to the salivary acini, and for the passage of saliva through the ductal system. This lag-time would be reduced at increased flow rates (Oliveby *et al.*1989a,c). The influence of flow rate, pH and plasma fluoride levels on salivary fluoride concentration has been investigated extensively by Oliveby and colleagues (1989a,b,c). Their conclusions were that the S/P ratio was influenced by the plasma fluoride concentration, but was unaffected by salivary flow rate or by salivary pH. This is in contrast to many other salivary ions, such as sodium, potassium, chloride, etc. which are influenced by both flow rate and salivary pH. In Figure 6.2 parotid saliva fluoride concentrations, following ingestion of a 3 mg oral dose of sodium fluoride, are plotted against time. Each value is the mean (S.E.) of the same four subjects as in Figure 6.1, at times up to 135 minutes after baseline. In accordance with the studies described above, the peak concentration occurred in saliva at 45-60 minutes, slightly later than the plasma peak recorded in Figure 6.1. In addition, the peak concentrations were markedly lower in parotid saliva than in plasma.

A number of animal studies have suggested a potential therapeutic role for duct saliva fluoride. Larsen & Mellberg (1977) showed that fluoride administered by gastric intubation to rats on a cariogenic diet had a cariostatic effect on erupted molars. The only oral fluoride exposure in this experiment was *via* recirculation in the saliva.

Figure 6.2 Mean (S.E.) parotid salivary fluoride concentrations versus time (minutes) following 3 mg oral dose of NaF (n=5).



Whitford & Schuster (1982) conducted a similar study in rats by infusing fluoride constantly into the bloodstream *via* an implanted, subcutaneous device. They noted a similar degree of caries reduction and also reported a slight elevation in the salivary fluoride concentration compared with that in control animals. These studies lend further support to the hypothesis that very low levels of fluoride can have a cariostatic effect, and also suggest that swallowed fluoride supplements such as tablets and drops may have some secondary topical, as well as systemic, mechanism of action.

6.3.2 Whole (Mixed) Salivary Fluoride.

The fluoride concentration in whole saliva derives from two major sources. These are the fluoride recirculating *via* duct saliva and the fluoride from topical agents placed in the oral cavity, and providing fluoride to plaque fluid, micro-organisms, salivary proteins and superficial enamel and exposed dentine. This variety of sources of whole saliva fluoride results in a much more complex pattern of accumulation, clearance and elimination than that seen in duct saliva.

One of the earliest studies published on the clearance of fluoride from the mouth was that of Aasenden, Brudevold & Richardson (1968), who investigated the levels of fluoride retained in whole, mixed saliva following topical application of either a 1.25% APF gel, a 0.5% APF gel, or an 0.2% NaF rinse. The findings of these authors included a total elimination time of 1 week for the 1.25% gel, of 4-6 days for the 0.5% gel and 'more than one day' for the mouth rinse. The delayed clearance of the fluoride from the oral cavity was attributed to the 'prolonged leaching of fluoride from the teeth'. They also noted that, in the absence of fluoride treatments, salivary fluoride levels were maintained at a constant level, and were not altered by a threefold increase in rate of flow. Another early study was that of Yao & Grøn (1970) who investigated the mixed and duct salivary fluoride concentrations in children and adults in fluoridated and non-fluoridated communities, and concluded that the higher

fluoride concentrations found in mixed saliva as compared with duct saliva were due to the inclusion of cellular debris and mucus, since the concentrations decreased when the mixed saliva was 'cleared' by centrifugation. Dental plaque and material scraped from the tongue was found to contain significant amounts of fluoride.

More recently, Dawes (1983) produced a mathematical model for the clearance of sucrose from the mouth, which has been modified and updated to apply to many other substances, including fluoride (Ekstrand, Lagerlöf & Oliveby,1986). In this model, the oral cavity is assumed to act as an incomplete siphon, into which saliva flows at a rate determined by the stimulating action of any tastant in the mouth, but with a basal secretion rate equivalent to the unstimulated flow rate. As saliva enters the mouth, the volume of saliva increases to a maximum value, deemed VMAX, at which point swallowing occurs. The salivary volume is immediately reduced to a residual level (RESID), when the cycle begins again. The model predicts that salivary clearance will take longer with a lower, unstimulated salivary flow rate; a higher initial concentration of material taken into the mouth; a higher residual volume, and a higher maximum volume before swallowing occurs. Much of this model has been confirmed experimentally (Lagerlöf & Dawes,1985; Billings *et al.*1988).

A major difference between fluoride and many other substances, such as sucrose, with respect to oral clearance, however, is that fluoride may react with tooth mineral or calculus. At concentrations less than 4 mmol/L, very small amounts of fluorohydroxyapatite, which is insoluble in saliva, may be formed. However, at higher concentrations, fluoride reacts with tooth mineral to form significant amounts of calcium fluoride (Leach,1959). Although saliva is unsaturated with respect to calcium fluoride, the latter dissolves only very slowly over days or weeks (Grobler, Ögaard & Rölla,1981), apparently due to surface adsorption of inorganic phosphate or pyrophosphate from saliva (Lagerlöf *et al.*1988). It may be, therefore, that calcium fluoride formed under such conditions, acts like a 'slow-release' device, and contributes to the prolonged intra-oral clearance time of fluoride. An updated

version of Dawes model factors in this ability of fluoride to interact with other substances and integuments in the oral cavity (Dawes,1987) and predicts, much more accurately than the original model, the very prolonged clearance time of high concentration topical fluoride agents.

Until recently, attempts to identify the intra-oral fluoride 'store' have been directed towards either the hard tissues of the mouth, enamel and dentine, and the calcium fluoride which may be deposited there during topical fluoride application, or towards the possible role of fluoride in dental plaque lying on these hard tissues. Undoubtedly, dental plaque contains high levels of fluoride [2.5 - 3.0 ng F/mg wet weight of plaque, (Duckworth *et al.*1994)], particularly in individuals who regularly apply topical fluorides (Tatevossian,1990; Vogel, Carey & Ekstrand,1992) and may contribute fluoride back into solution, particularly at reduced pH. However, since the publication of one paper over 20 years ago (Gabler,1968) and the observation by Yao & Grøn in 1970 that tongue and cheek scrapings had considerable quantities of fluoride, the potential role of the oral soft tissues in absorbing and delaying the clearance of fluoride from the mouth has been largely overlooked.

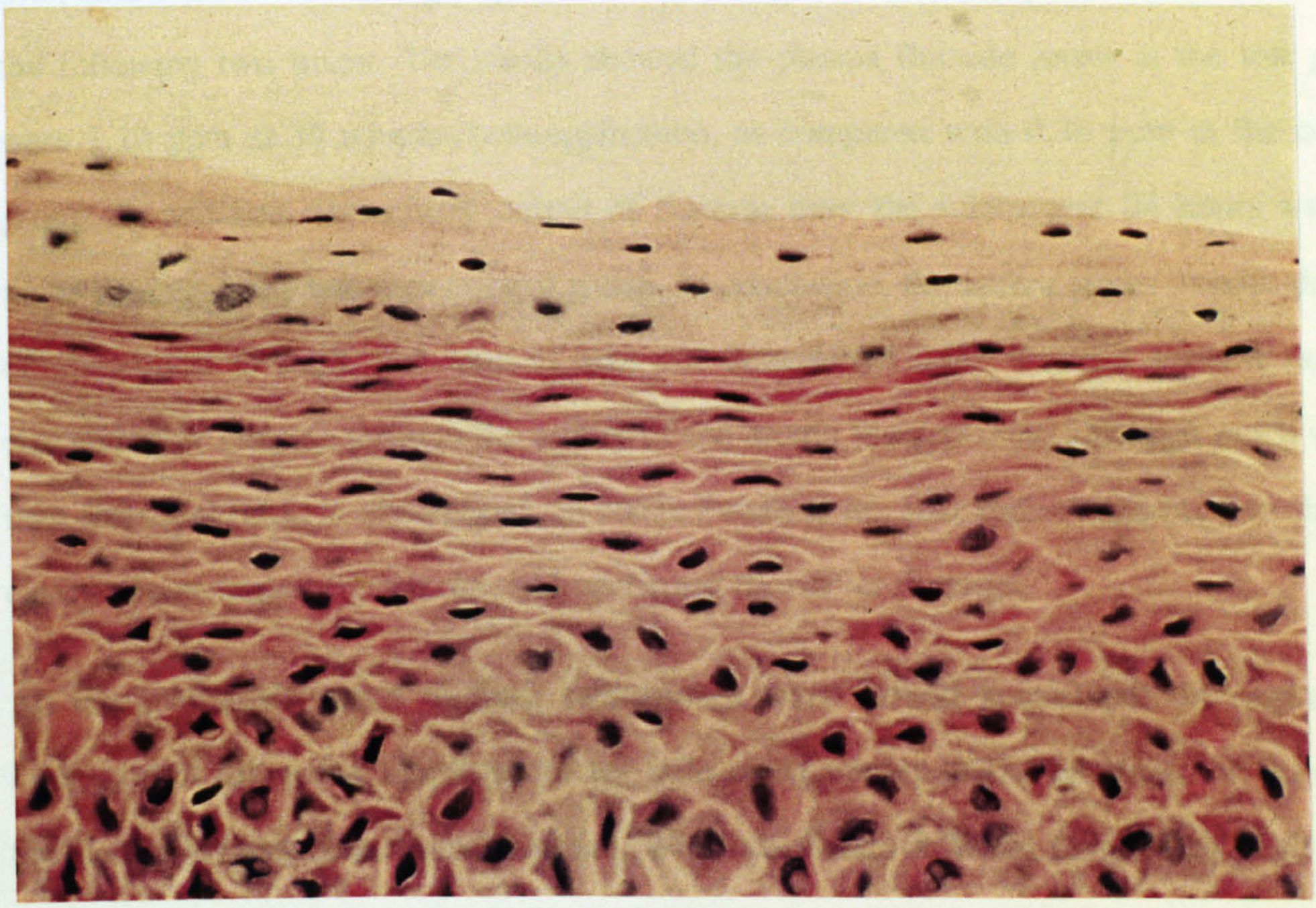
6.4 The Oral Mucosa.

The oral mucosa in different regions of the mouth varies considerably in structure, but can be divided broadly into two categories :- the keratinised masticatory mucosa, which includes the hard palate, dorsum of the tongue and the gingivae; and the non-keratinised lining mucosa (see Figure 6.3), which covers the remainder of the oral cavity (Squier, Johnson & Hackemann,1975). In addition, there are variations in the thickness of the epithelium and the components of its submucosa, such as the presence or absence of minor salivary glands and blood vessels. Studies *in vivo* on the permeability of human oral mucosa (Kaaber,1971a; Kaaber,1971b) have shown a difference in regional permeability to water between the palatal and buccal areas, the keratinised palatal surface having a much lower permeability. In studies

on porcine oral mucosa, Squier & Hall (1985), found that the floor of the mouth was significantly more permeable than any other site in the oral cavity, whilst the keratinised gingival epithelium was closer, in terms of water permeability, to epidermis. Studies on the permeability of oral mucosa from cadavers (Lesch *et al.*1987), also showed the floor of the mouth and the lateral borders of the tongue were the most permeable regions. The relative importance of a given site in absorption will, however, depend on its total surface area as well as its permeability. In a widely quoted study by Collins & Dawes (1987), the total surface area of the mouth, including the teeth, averaged 214.7 cm^2, with approximately 80% of this area covered by mucosa. This obviously represents an extensive region for potential absorption of compounds from the oral cavity.

The ability of substances to penetrate and cross the oral mucosa has been utilised to advantage in both medicine and dentistry, for sublingual administration of such compounds as glyceryl tri-nitrate, and topical anaesthetics such as lignocaine. In addition, *in vitro* studies have demonstrated that very large molecules such as albumin and transferrin (Brandtzaeg & Tolo,1977), and dextran and inulin (Siegal,1981) can traverse the oral mucosa. It seems likely, therefore, that a compact ion such as fluoride may quite easily become adsorbed/absorbed *via* this route, and either be released back into the oral cavity when conditions are favourable, or eventually enter the central plasma compartment for recirculation. This hypothesis is explored further in the remainder of this chapter.

Figure 6.3 **Photograph of the histology of oral, non-keratinised (lining) mucosa (H&E, x 100 magnification).**



6.4.1 Fluoride Absorption by the Oral Mucosa.

As mentioned in the previous section, Gabler published the results of a study in 1968, which had investigated the absorption of fluoride through the oral mucosa of rats. Two groups of animals were used in the study, one to monitor changes in plasma fluoride concentrations and the other to monitor the total urinary output of fluoride during the 24 hour period following the experiment. Both groups of rats had oesophageal ligation under general anaesthesia, prior to the application of either 2% sodium fluoride solution (Test group) or 2% NaCl solution (Control group) to the oral mucosa. Plasma samples were collected over the following two hours. The results showed the plasma fluoride levels in the test group were 1.10 ppm at 30 minutes post-application, as compared with 0.30 ppm in the control group. In addition, the urinary output of fluoride over the subsequent 24 hours was 6-7 times greater in the test than control group. In conclusion, the author states "whilst the rate of absorption through oral mucosa was not determined.....the findings suggest that the absorption of fluoride through the tissues of the mouth is significant, although much slower than intestinal fluoride absorption". In 1978, Patten *et al.* also applied solutions to the oral cavity of oesphageal-ligated rats, and confirmed the earlier findings.

All the solutions in used in these two studies were around neutral pH. In response to findings that pH was a significant factor influencing transepithelial migration of fluoride in the kidney, urinary bladder and stomach, Whitford, Callan & Wang (1982) published details of a study on the influence of pH on fluoride absorption through hamster cheek pouch (keratinised) epithelium. Here, three different concentrations of fluoride solution were used, 20, 200 and 1000 ppm, and each fluoride concentration was buffered to each of three pH's, 2.4, 3.8 and 6.9. The results showed significant systemic uptake of fluoride from all three fluoride concentrations. In fact, the fractional absorption of fluoride was independent of the solution fluoride concentration, which indicates first order absorption kinetics i.e. diffusion. However, reducing the pH had a very significant effect on the amount of fluoride absorbed

(19.6% of total dose for pH 2.68 versus 4.2% of total dose for pH 6.88, $p < 0.001$). The authors conclude that the transepithelial migration rate of fluoride is inversely related to solution pH, and that fluoride most likely permeates biological membranes as the undissociated acid, HF.

A subsequent *in-vitro* study (Duckworth & Jones,1989), using porcine oral mucosa, confirmed that uptake of fluoride by soft tissue is enhanced markedly by a reduction in solution pH, and also concluded that uptake of neutral HF by oral soft tissue was more pronounced than of negatively charged fluoride ions.

One of the first studies to look at the possible role of human oral soft tissue in fluoride clearance was that of Zero *et al.,* (1990), which compared the fluoride retention of a variety of topical agents in both dentate (hard and soft tissue) individuals and edentulous (soft tissue only) individuals. They found there was no significant difference between the fluoride clearance patterns for the dentate and edentulous individuals, when using either a fluoride dentifrice (1100 ppm) or a fluoride rinse (226 ppm). The only significant difference detected was for the fluoride gel (5000 ppm), delivered in custom trays, and here the edentulous group had significantly higher fluoride levels at the one hour time interval (3.04 ppm versus 0.64 ppm). The authors' conclusions are that the oral soft tissues may be the primary site of fluoride retention during the early phase of fluoride clearance from the mouth.

The following sections detail further investigations undertaken as part of this thesis into the role of oral soft tissues in fluoride clearance.

6.4.2 A Reproducibility Study.

The significant issues to be addressed at the start of these soft tissue studies were firstly, to identify the most suitable site in the oral cavity for soft tissue sampling, and secondly to devise a method that would allow multiple sampling without causing undue discomfort to

the subjects or excessive disruption to the normal mucosal architecture. Following a number of pilot efforts, it was decided that the buccal mucosa presented a sufficiently large area of relatively homogenous tissue, and was likely to be less contaminated by food debris than the dorsum of the tongue, the other potential site. With respect to sample collection, it was deemed important to remove at least the most superficial two or three cellular layers, in order to ensure the sample was truly intra-epithelial, and not just the salivary film and cellular debris from the surface (see Figure 6.4). In order to achieve this, the collecting instrument required to have a relatively sharp edge, without being able to actually incise the tissues. In addition, this instrument had to be very light, to facilitate the accurate weighing of the sample on a five-figure balance, and it had to be virtually inert, to avoid contamination of the sample, or the subsequent analytical techniques. The instrument finally selected was an Odus cervical matrix (see Figure 6.5), which was originally intended for maintaining dental restorative materials *in situ* during polymerisation. These matrices are made of cellulose acetate, and proved to be inert in the buffer solution (TISAB) which was used in this study.

Figure 6.4 **Photograph of the buccal mucosa epithelial cell sample stained by the Papanicolaou technique.**

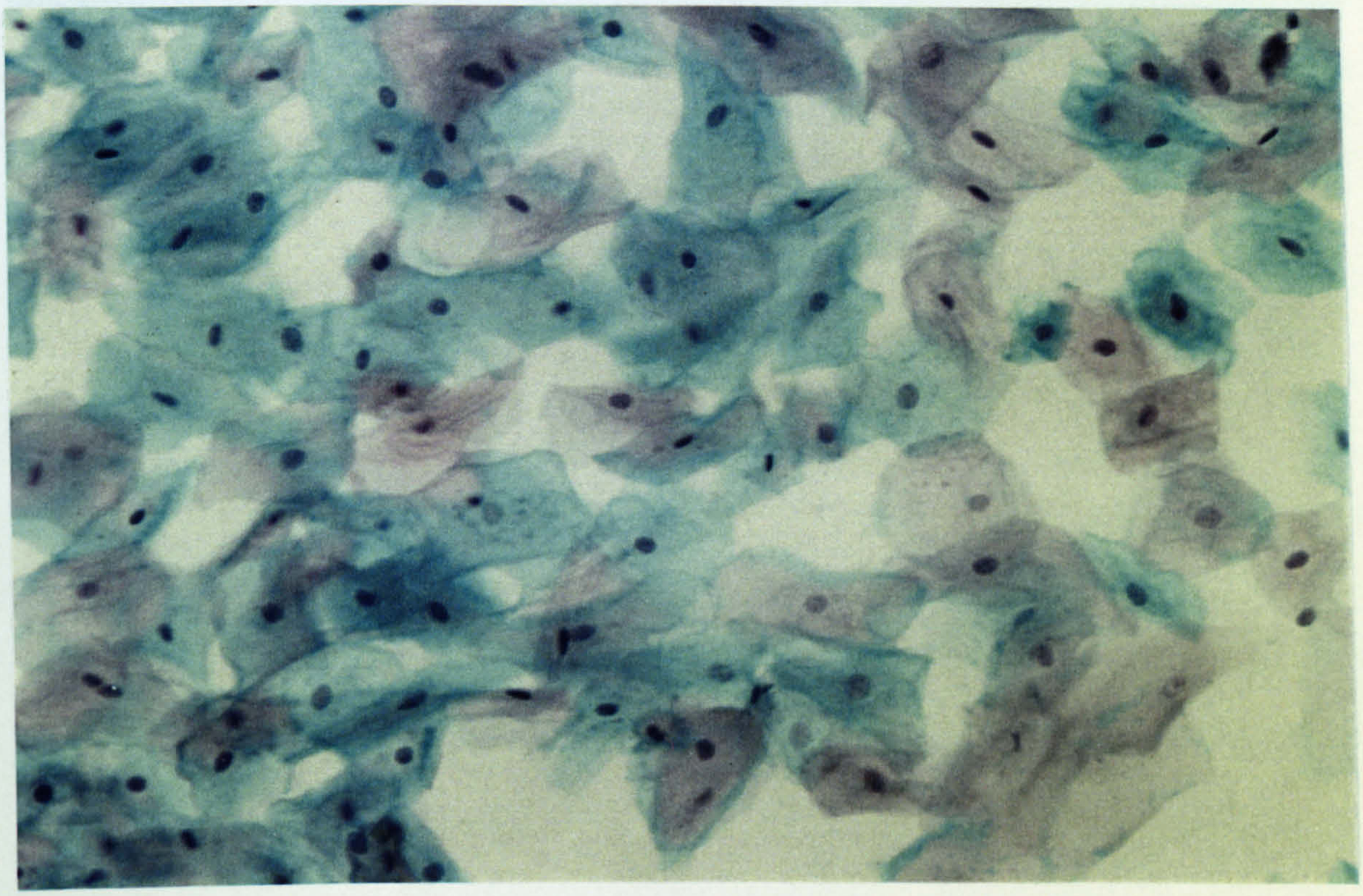
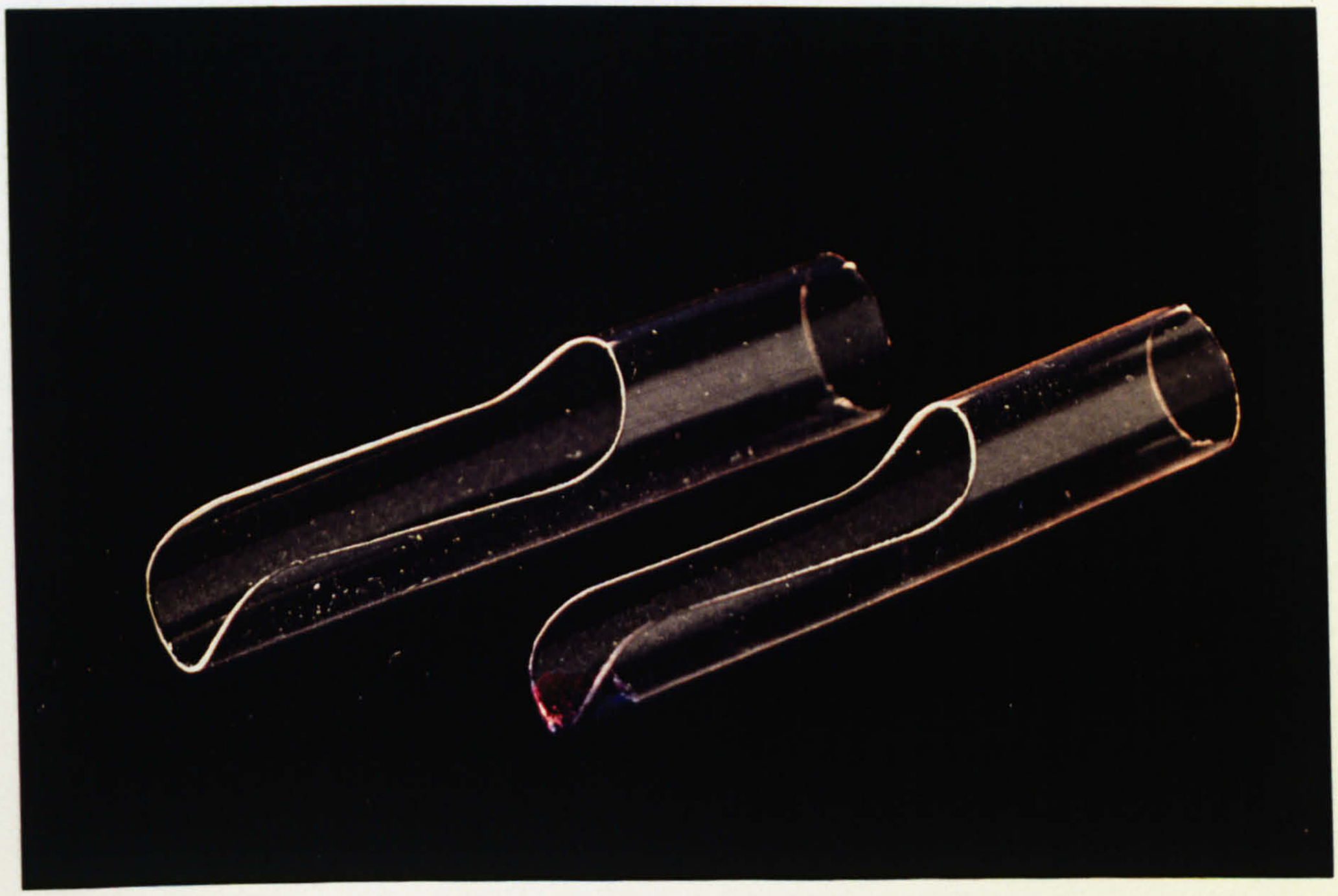


Figure 6.5 **Photograph of *Odus* cervical matrices used to collect buccal mucosa samples.**



It was not possible to repeatedly sample the same site on the buccal mucosa, as the removal of the superficial cell layers was likely to affect the mucosal permeability. It was necessary, therefore, to establish the level of reproducibility which could be expected if different sites on the buccal mucosa were sampled sequentially.

A total of six volunteers were recruited to establish the reproducibility of this technique. All were adults who routinely used fluoridated dentifrices and who had participated in other studies reported in this thesis. The volunteers were asked to refrain from using any fluoridated product for the 24 hours preceding the experiment. Each volunteer was allocated a time slot, either baseline, t+5, t+15, t+30, t+45 or t+120. Sufficient matrices (6 samples x 6 subjects) were carefully weighed on a Sartorius MC1 balance (*Sartorius,* Gottingen, Germany). Bijou bottles were prepared for each sample, containing a solution of 1/1 vol./vol. deionised water and TISAB acetate buffer. The volunteer assigned to provide the baseline samples then had superficial mucosal cells removed from three distinct areas on both the left and right buccal mucosa (see Figure 6.6). The samples of mucosal cells could be clearly seen by the naked eye. All the samples were weighed quickly whilst on the matrix, and then transferred to the deionised water/buffer solution and sealed. The weight of the sample was determined by subtraction of the matrix weight from the matrix plus sample weight. All samples were stored at -16°C until required for analysis.

The remaining five volunteers then rinsed for 1 minute with 10 ml of an 0.2% NaF solution and then expectorated. At each of the times t+5, t+15, t+30, t+45 and t+120, one of the subjects had six buccal mucosal samples collected from distinct areas of the right and left buccal mucosa. At all times, attempts were made to ensure sufficient material was removed to facilitate the subsequent fluoride analysis and the weighing and sealing procedure was carried out as rapidly as possible to minimise any possible sample evaporation. Prior to analysis, all samples were brought to room temperature, vortex mixed for 30 seconds to redisperse the high molecular weight material, and the fluoride content determined in

accordance with the procedures in Appendix 3.4.

The results of this reproducibility study are shown in Table 6.1 and Figure 6.7. The calculation of mucosal fluoride concentration assumed a density of 1 for the mucosal sample, i.e. weight was equal to volume.

In spite of endeavours to the contrary, two samples were of very low weight (< 2 mgs), and as discussed in relation to plaque sampling in Chapter 4, low weight samples tend to provide unpredictable results. If these two samples are removed from the equation, the reproducibility of the technique varies from 5.6% at t+5 to 2.5% at t+120. The highest coefficient of variation corresponds to the earliest timepoint, when the finite amount of time required to collect the six samples is likely to have its greatest effect. Of interest here also, are the relatively high baseline mucosal fluoride levels (~9-10 ppm), noted in the subject who provided the baseline samples. Overall, it was concluded that the different sites could be used sequentially to investigate fluoride clearance up to two hours after rinsing.

Figure 6.6 **Photograph of the buccal mucosa, with three sample collection sites on the left cheek outlined.**

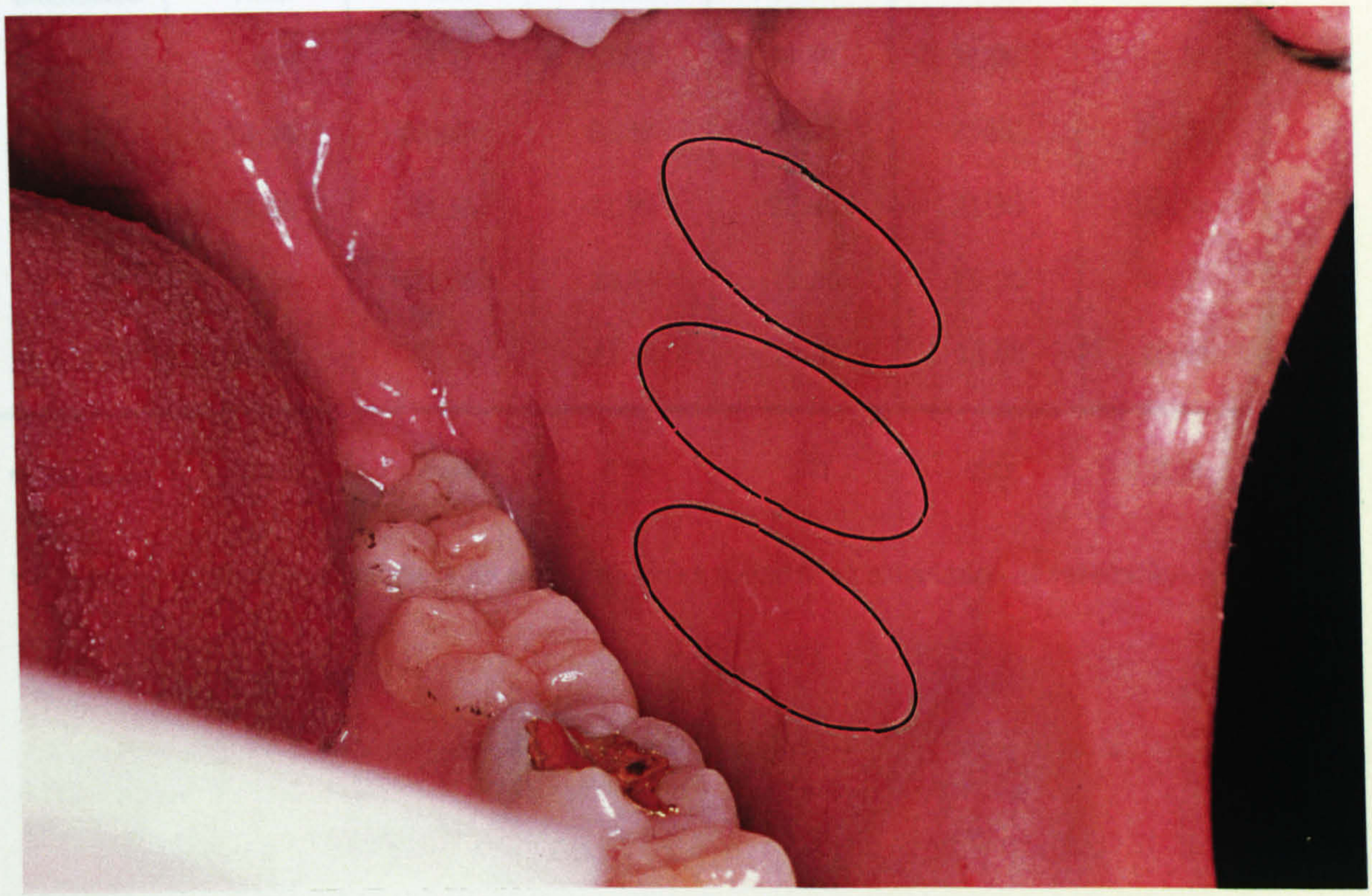
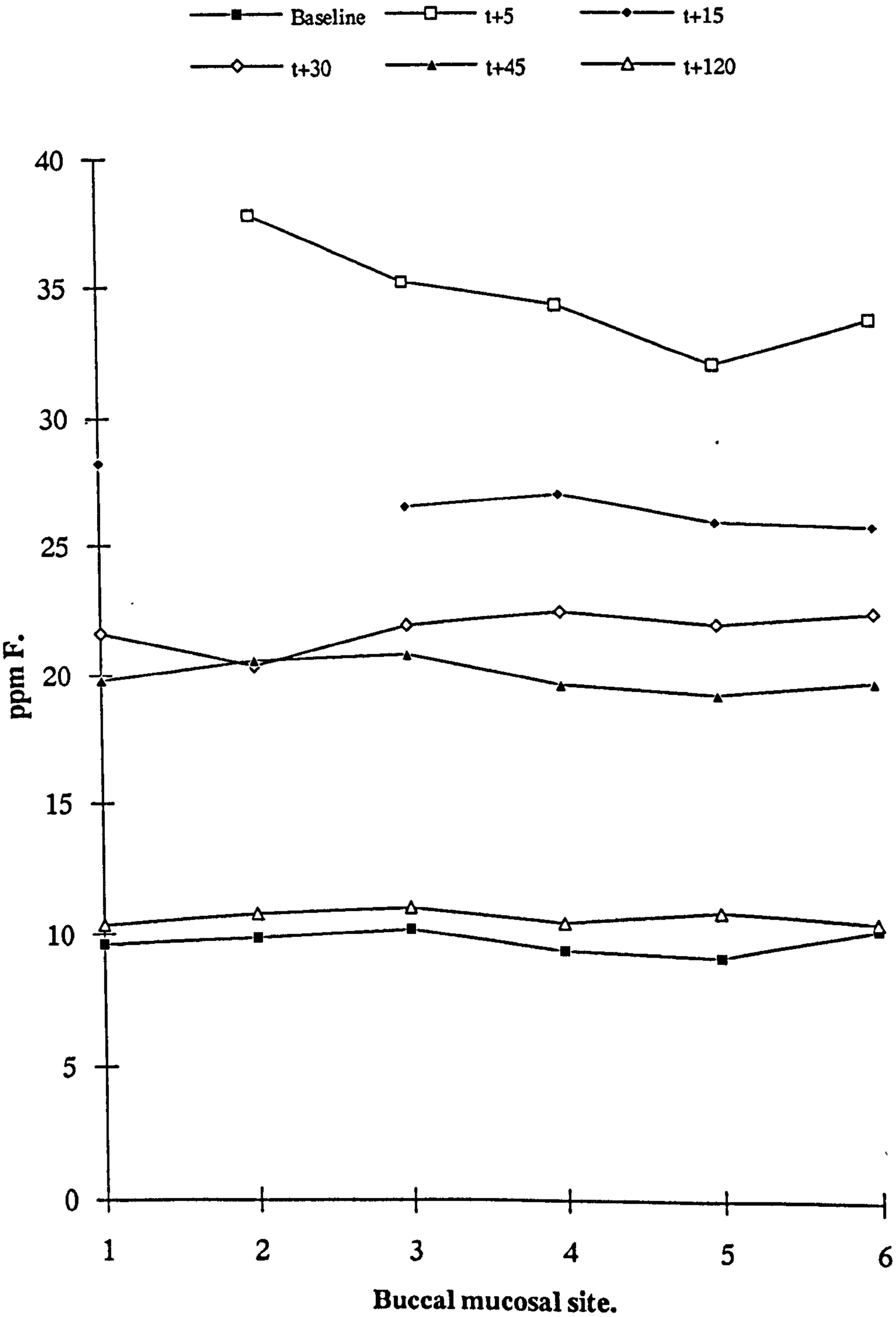


Table 6.1 **Buccal mucosal fluoride values (ppm F) following 1 minute rinsing with 0.2% NaF - Six sites per subject at one time point.**

Time	Right 1	Right 2	Right 3	Left 1	Left 2	Left 3
Baseline	9.62	9.85	10.20	9.37	9.10	10.15
t + 5	51.06*	37.28	35.20	34.30	32.00	33.70
t + 15	28.20	65.60*	26.50	27.00	25.90	25.70
t + 30	21.60	20.35	21.90	22.40	21.90	22.30
t + 45	19.80	20.56	20.80	19.60	19.20	19.7
t + 120	10.35	10.76	11.02	10.45	10.80	10.40
%Variation	4.48	5.64	3.75	3.41	2.99	2.52

* two samples of < 2 mgs weight (1.88 and 1.28) are excluded from calculation of variability.

Figure 6.7 Variation in buccal mucosal fluoride levels at six different sites.



6.4 3. A Mucosal Fluoride Clearance Study.

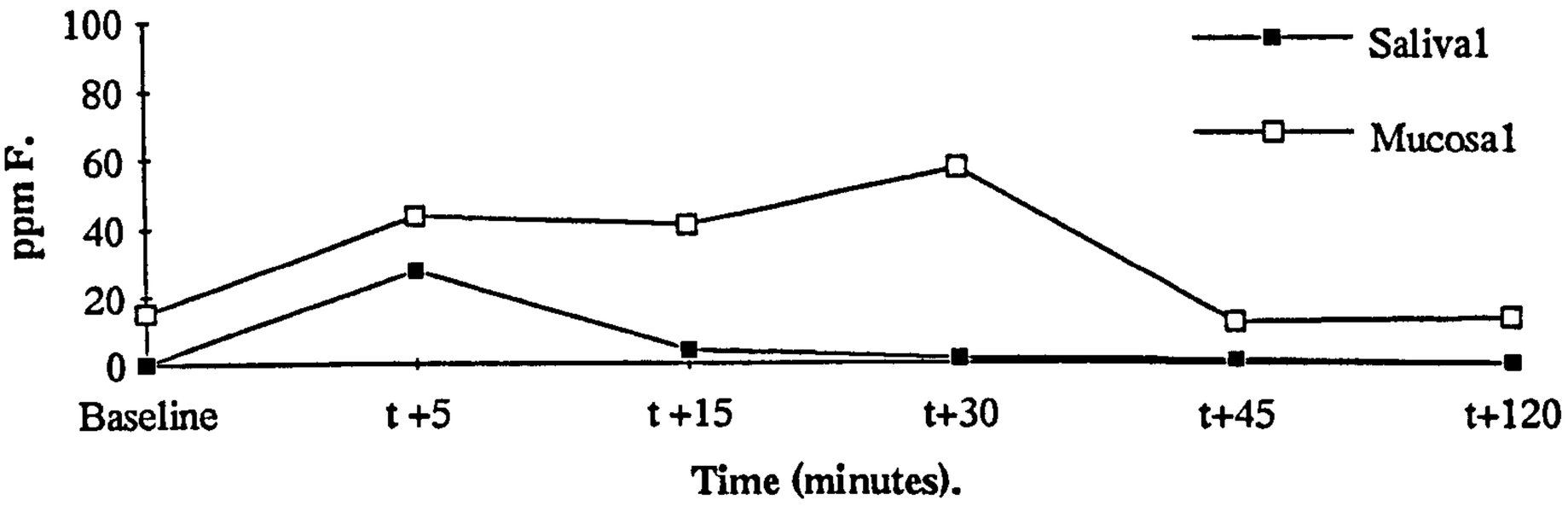
With variability of the technique within acceptable limits, a clearance study was undertaken. For this, five different volunteers were selected, as advice from in-house pathologists suggested that there should be at least weekly intervals between consecutive sampling of the same site, to ensure the normal mucosal architecture was re-established. This factor limited the study to six potential mucosa samples per volunteer.

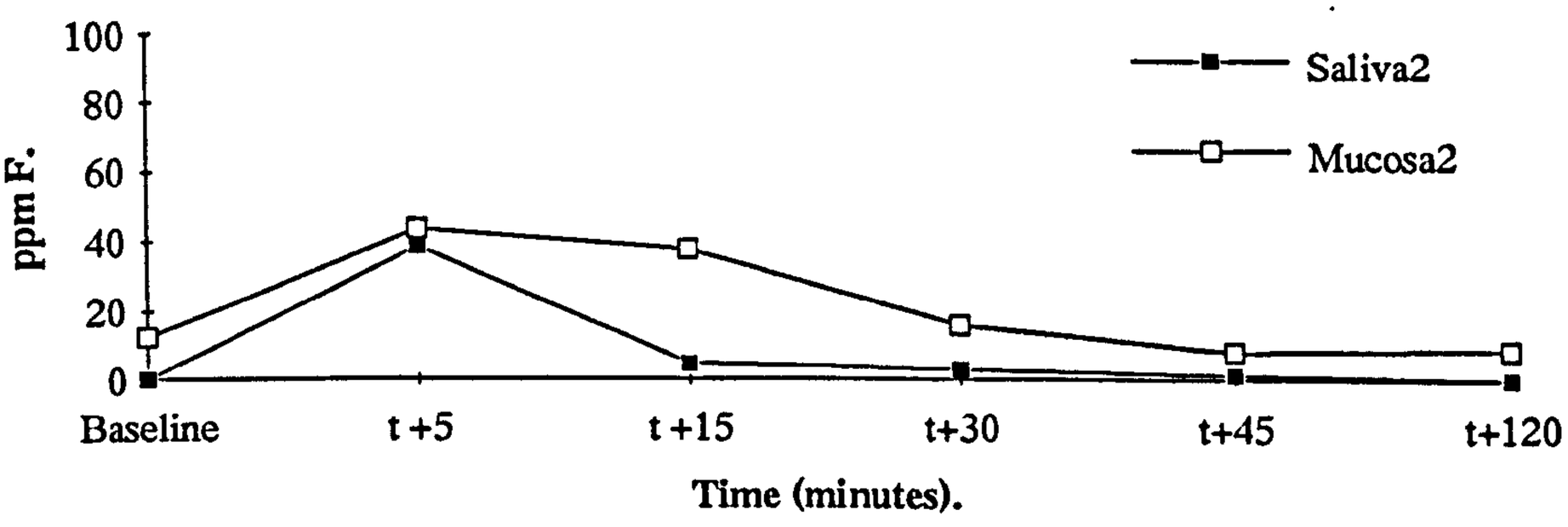
As before, baseline samples were collected from all volunteers, following a 24 hour period avoiding use of topical fluoride agents. For this study, mixed saliva samples were collected at the same times as the mucosal samples using the technique described in Chapter 3. All volunteers then rinsed with 10 ml 0.2% NaF solution (pH 5.8) for 1 minute and then expectorated. Mucosal samples were collected at 5, 15, 30, 45 and 120 minutes after rinsing (R1, R2, R3, L1, L2 and L3) and saliva samples were collected immediately following each mucosa sample. The samples were weighed, buffered and stored as described earlier. Fluoride analysis was undertaken as detailed in Appendix 3.4. The results of this clearance study are given in Table 6.2 and Figures 6.8 and 6.9.

Table 6.2 **Mixed saliva *(italics)* and buccal mucosa clearance (ppm F) from 5 subjects following 1 minute rinsing with 0.2% NaF.**

Time	Subject 1		Subject 2		Subject 3		Subject 4		Subject 5	
Baseline	*0.05*	14.9	*0.09*	12.3	*0.03*	3.64	*0.02*	6.48	*0.02*	9.32
t +5	*27.5*	43.5	*38.6*	43.4	*40.0*	98.2	*103.0*	39.4	*96.2*	56.3
t + 15	*3.5*	40.9	*4.6*	37.4	*4.9*	54.3	*12.2*	31.6	*31.9*	48.2
t + 30	*1.4*	57.3	*2.7*	16.0	*1.92*	36.4	*8.5*	2.7.7	*14.2*	37.0
t + 45	*1.1*	12.5	*1.4*	8.2	*1.5*	13.3	*4.0*	14.9	*7.1*	18.6
t + 120	*0.3*	13.5	*0.2*	8.7	*0.6*	3.5	*0.8*	8.9	*0.62*	11.3

Figure 6.8. **Fluoride clearance from mixed saliva and buccal mucosa following 1 minute rinsing with 0.2% NaF.**





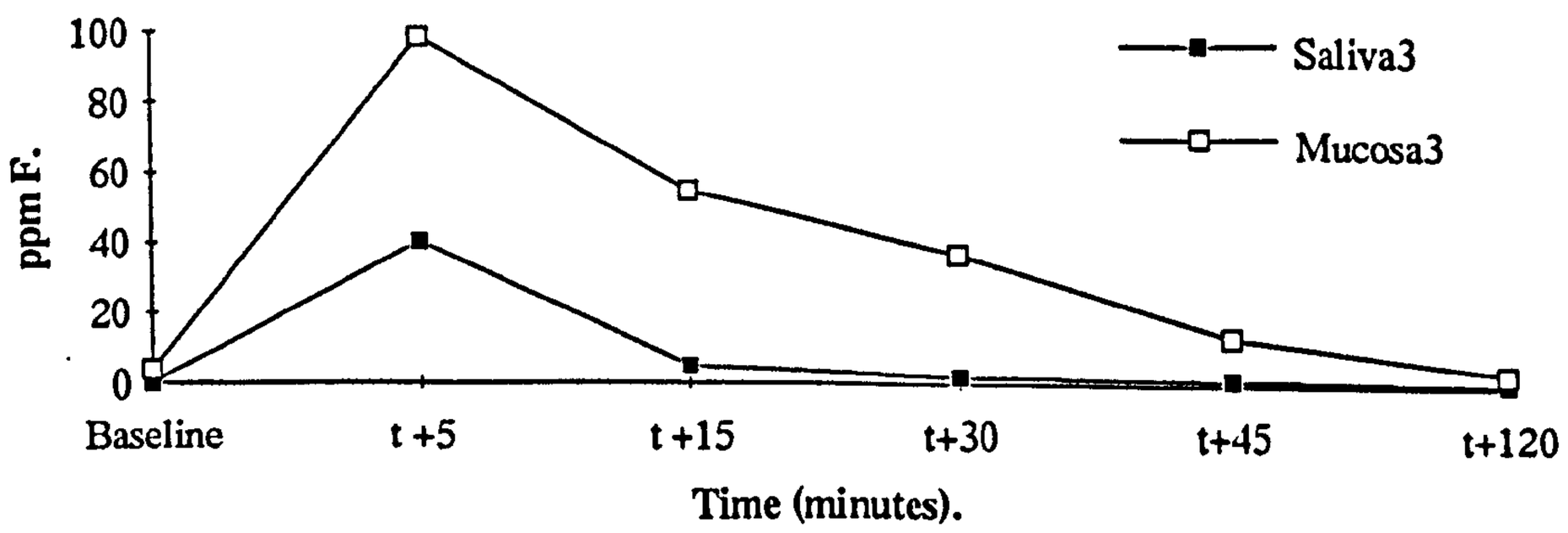
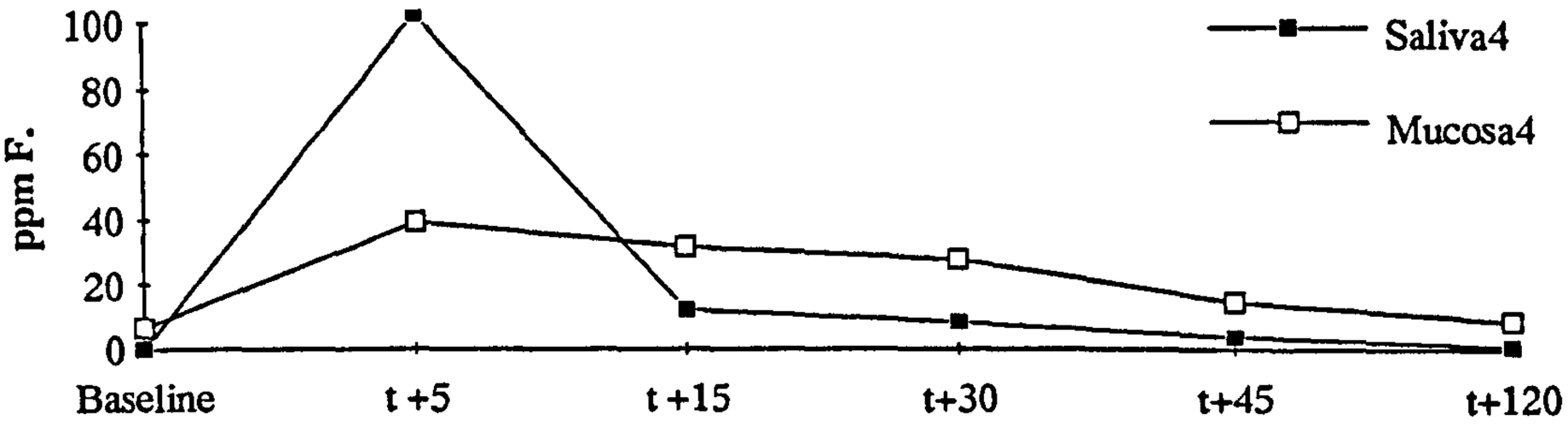


Figure 6.8 (cont). **Fluoride clearance from mixed saliva and buccal mucosa following 1 minute rinsing with 0.2% NaF.**



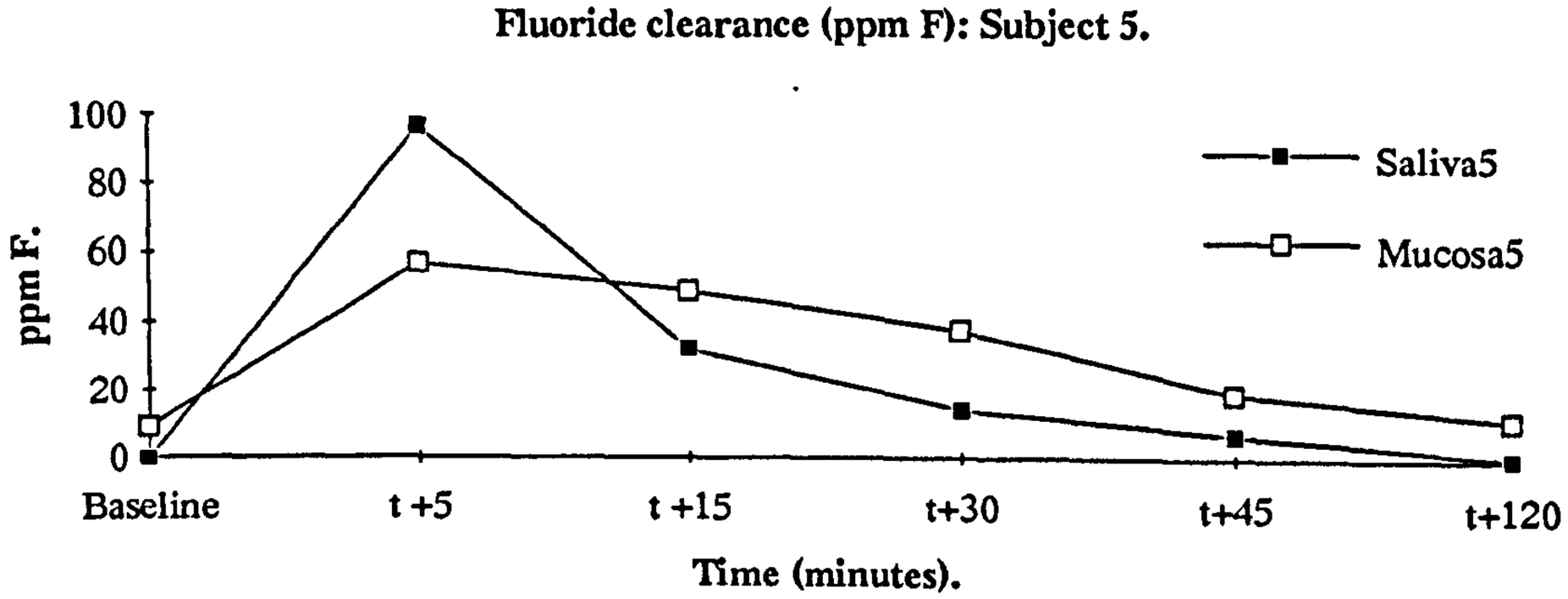
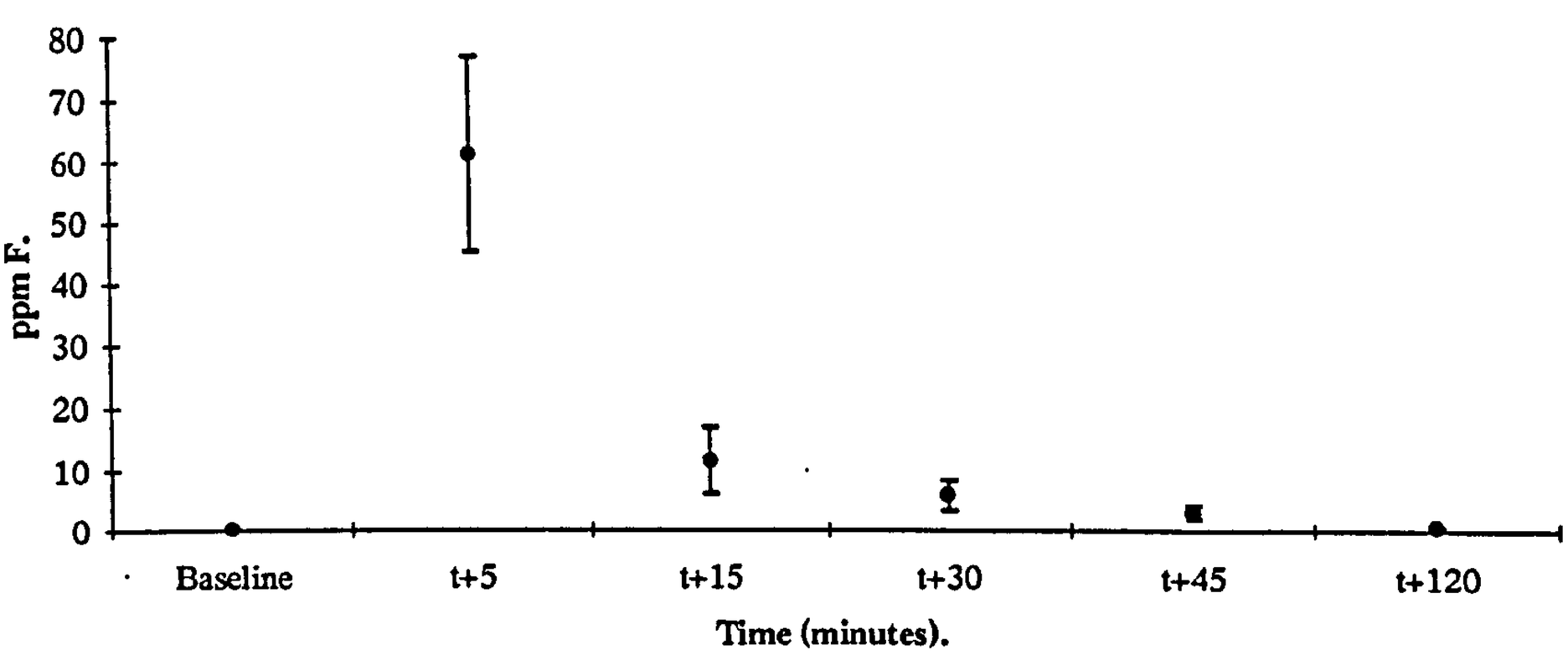


Figure 6.9 **Mean (S.E.) mixed saliva and buccal mucosa fluoride clearance following rinsing for 1 minute with 0.2% NaF (n=5).**



In Figure 6.8, the clearance pattern for each subject is presented separately. In all cases there is a rapid rise in salivary fluoride from a low baseline level (mean 0.04 ppm), with the highest fluoride levels recorded at five minutes. From this peak level, there is a fairly rapid decline towards the 15 minute time interval, and then a more gradual decline thereafter. Whilst this pattern is reproduced in all the subjects, the peak salivary fluoride concentrations varied considerably (mean 61.06 ppm, S.E. 15.9).

The baseline buccal mucosa values varied considerably, (mean 9.34 ppm, S.E. 2.01) and were much higher than the corresponding saliva levels. In all five subjects, the mucosa fluoride levels also rose rapidly from baseline to the five minute sample time and for four of the five subjects, the peak fluoride concentration occurred at this time. In Subject 1, however, a higher value was obtained at the 30 minute time point, although this result is from a sample with a borderline acceptable weight (2.1 mg) and may have been artificially elevated. In general, there was a steady decline in mucosal fluoride after the five minute sample, although it appears the decline is much slower than that for saliva, as evidenced by the much shallower slope of the clearance curve at the later time points.

In Figure 6.9, the mean (S.E.) salivary and buccal mucosa fluoride levels are plotted for all five subjects. As a group, it is evident that the mucosal fluoride levels remain elevated for considerably longer than the those in saliva.

This study provides evidence to support the concept of fluoride adsorption to soft tissue during periods of high concentration in the oral cavity. In addition, the elevated baseline levels found here and in the reproducibility study, and the slow decline in fluoride concentration following the topical application, also support a potential role for the oral soft tissues as an intra-oral reservoir of fluoride which has the potential to resupply saliva as the levels therein decline.

6.4.4 A Fluoride Release Study.

In order to ascertain whether or not the fluoride identified in/on the oral mucosa following topical fluoride application could be released back into solution in saliva, a fluoride release experiment was devised. Once again, five subjects were identified, and here they were requested to use only a non-fluoridated dentifrice for the two weeks prior to the study, in an attempt to reduce the level of fluoride accumulated in the mouth. A modification of the Carlson-Crittenden parotid saliva collection cup (Carlson & Crittenden,1910) was constructed (see Figure 6.10), which had, in addition to the normal suction and outlet apertures, an inlet channel which enabled the introduction of solutions into the inner chamber of the cup. The cup device was applied to the buccal mucosa of each subject (one subject/day) and held in place with suction maintained via a three-way valve (for the assembled apparatus see Figure 6.11). This allowed the subjects to move around freely during the course of the experiment. An aliquot of distilled, deionised water (vol. 400µls) was then introduced into the inner chamber of the cup, now overlying a delineated area of mucosa. This solution was left *in situ* for 2 minutes, and evacuated from the cup for storage at -16°C. The collecting cup was then removed from the mouth and the subject rinsed with 10 ml of 0.2% NaF solution for 1 minute. Following expectoration, the collecting cup was re-seated in the same location as before. This accurate re-seating was facilitated by the slight, and temporary, erythema produced on the mucosa by the suction. (see Figure 6.12 for collecting device *in situ*). Aliquots of distilled, deionised water were introduced at the following times: 5, 20, 40, 60 90 and 120 minutes, and left *in situ* for 2 minutes each.

Following evacuation from the collecting cup, the samples were stored at -16°C, until required for analysis.

Figure 6.10 **Photograph of the modified Carlson-Crittenden collecting cup, with an additional aperture in the central collecting chamber.**

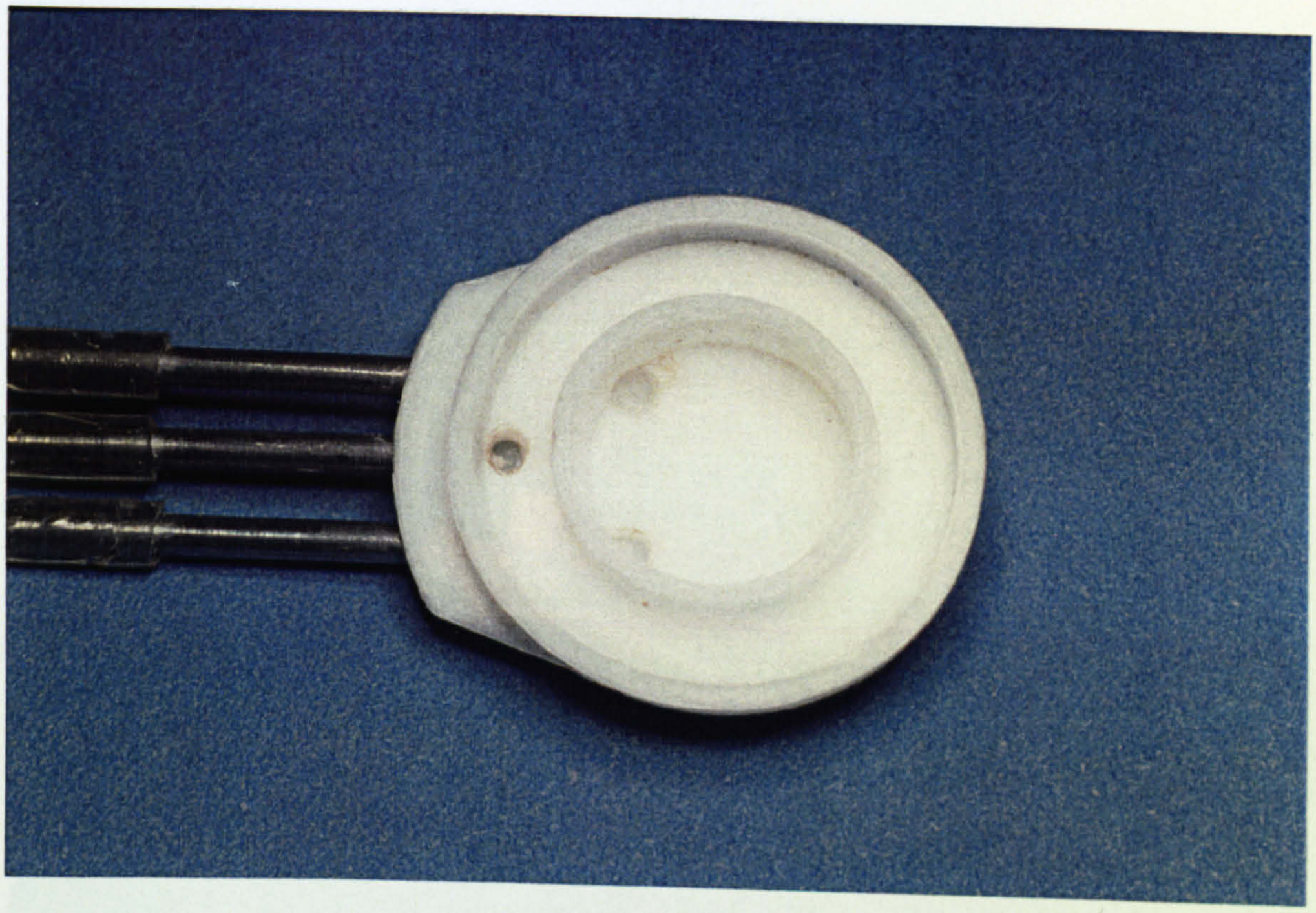


Figure 6.11 **Photograph of the assembled sample-collection apparatus, with the modified Carlson-Crittenden cup attached to a three-way valve and a 10 mL syringe to provide suction.**

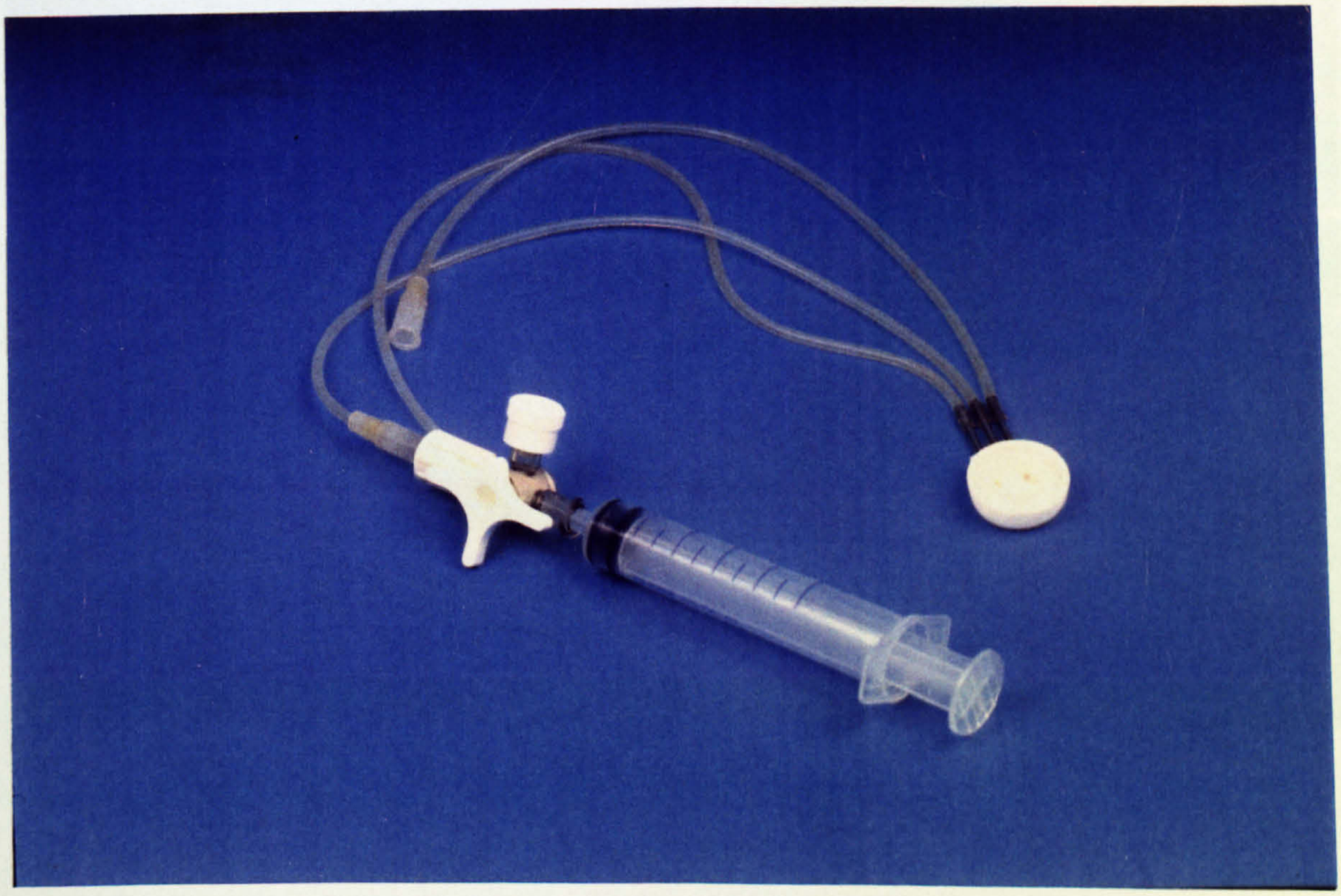
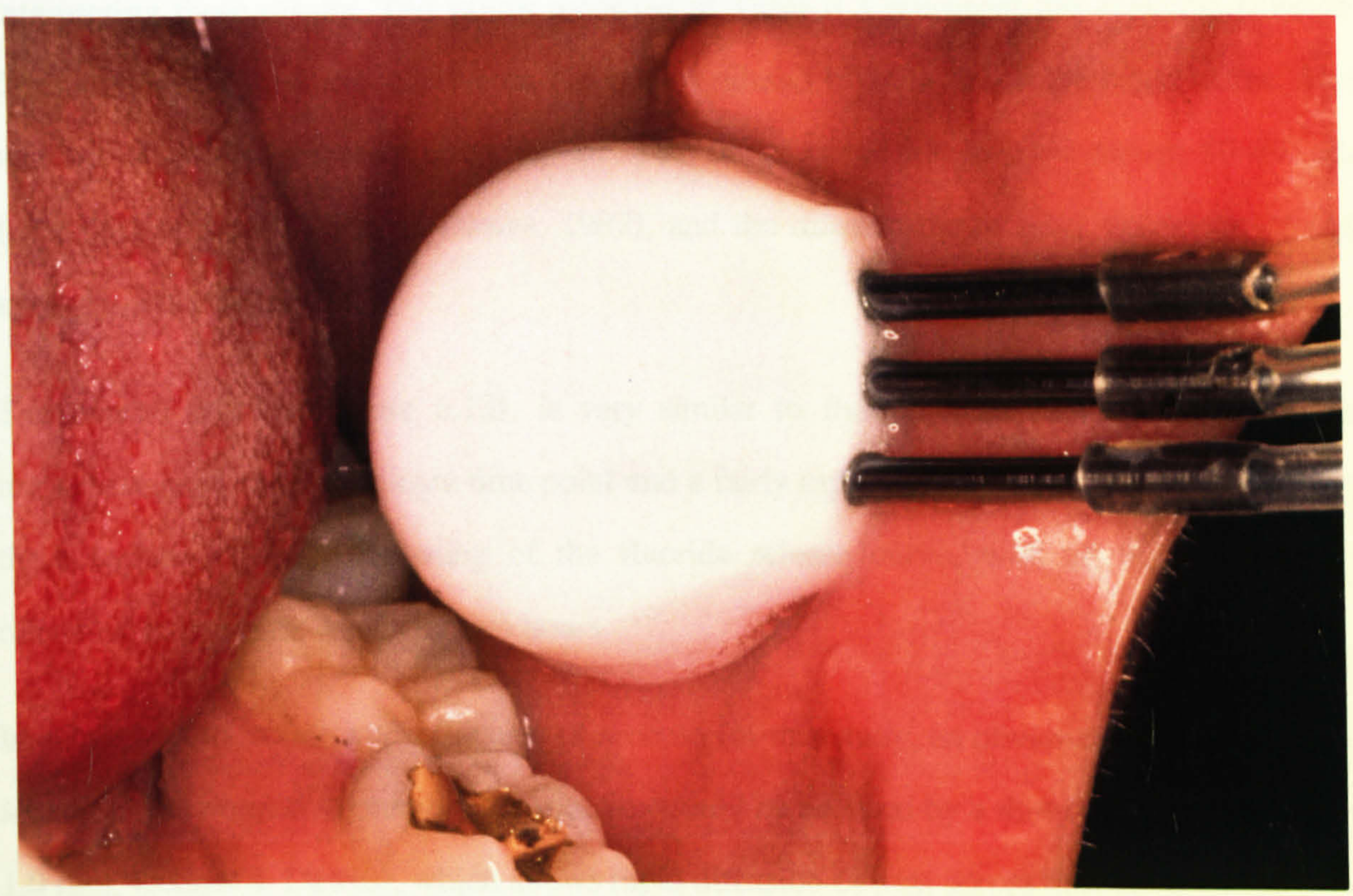


Figure 6.12 Photograph of the modified Carlson-Crittenden cup *in situ*.



All samples were analysed on the same day, after gradually being brought to room temperature. An equal volume of acetate buffer (TISAB) was added and the measurement procedure undertaken as in Appendix 3.4. The results of this study are given in Table 6.3 and Figure 6.13.

Whilst the actual levels reported here are much lower than in the previous two studies, (maximum value 0.6 ppm F), there is a significant dilution factor to be considered when interpreting these results. The values recorded in Table 6.3 represent the available fluoride from a circumscribed area of mucosa (64 mm^2), diluted by 400 µls of deionised water. The volume of saliva normally covering an area of mucosa this size is about 30-40 µls (extrapolated from Collins & Dawes, 1987), and the dilution factor here, therefore, would appear to be at least x10.

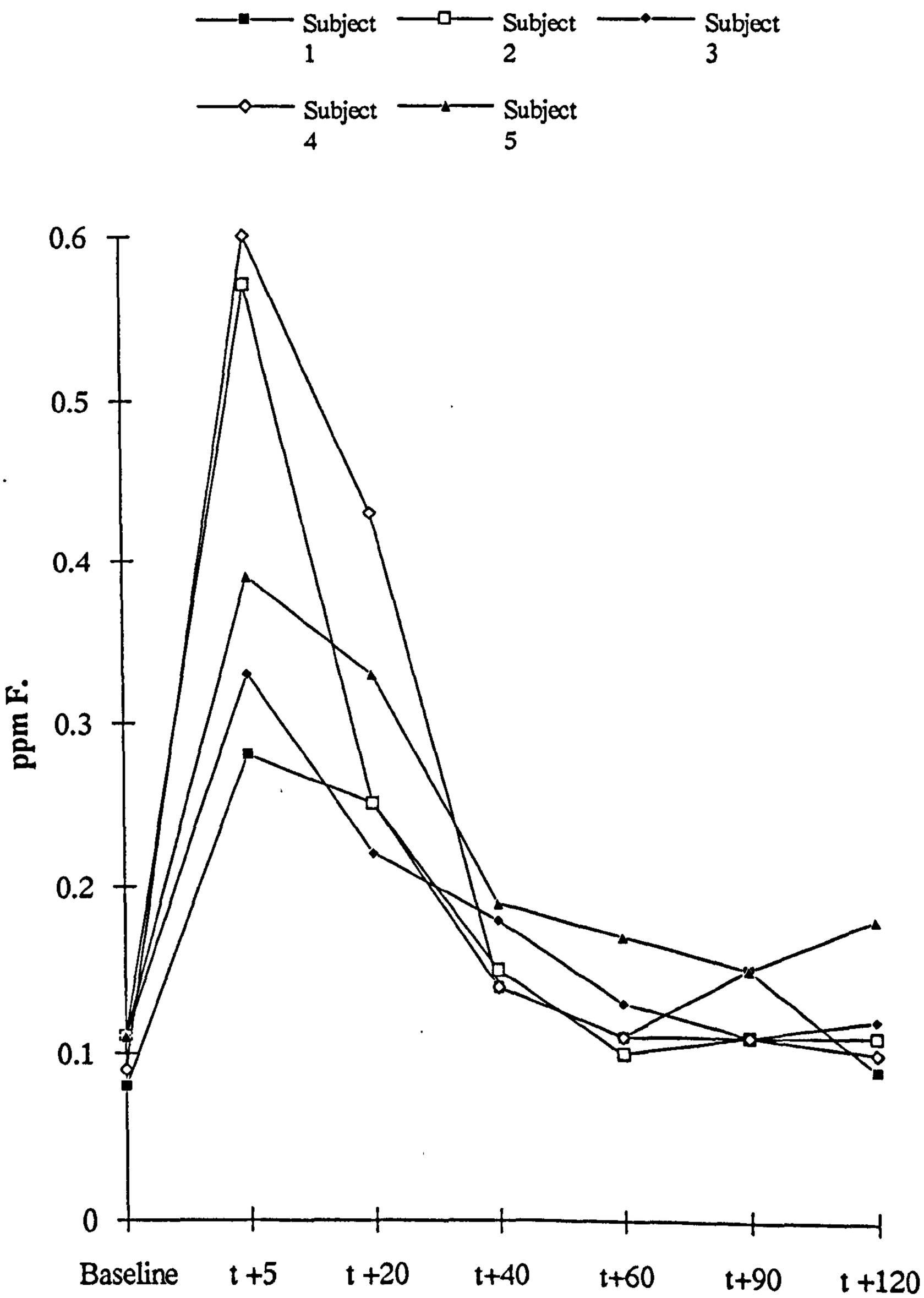
The overall pattern (Figure 6.13), is very similar to the previous two studies, with an immediate peak at the 5 minute time point and a fairly rapid decline thereafter. After 1 hour, there appears to be a flattening of the fluoride release curve, with all but one subject returning to pre-rinse levels by two hours.

In an attempt to isolate a specific area of mucosa for this study, an obviously artificial situation was created. Normally, the mucosa would be covered by a thin film of saliva, (0.7 - 1.0 mm, Collins & Dawes), which allows rapid diffusion of ions across the saliva:tissue interface, and which moves at variable rates across the tissues of the oral cavity, distally towards the oro-pharynx (Dawes,1993). In this study, however, a relatively large volume of fluid was placed in contact with the oral mucosa for a short period of time (2 minutes), and then removed in its entirety, to be replaced by a completely fresh solution a variable length of time later. This re-application of completely new solution would create an artificially steep concentration gradient, and may have resulted in a much faster release of fluoride from the soft tissues than would normally occur.

Table 6.3 **Fluoride concentrations of samples (ppm) washed from the buccal mucosa following 1 minute rinsing with 0.2% NaF.**

Time	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5
Baseline	0.08	0.11	0.11	0.09	0.11
t + 5	0.28	0.57	0.33	0.60	0.39
t + 20	0.25	0.25	0.22	0.43	0.33
t + 40	0.14	0.15	0.18	0.14	0.19
t + 60	0.11	0.10	0.13	0.11	0.17
t + 90	0.15	0.11	0.11	0.11	0.15
t + 120	0.09	0.11	0.12	0.10	0.18

Figure 6.13 Fluoride concentrations (ppm) of samples washed from the buccal mucosa following 1 minute rinsing with 0.2% NaF.



In addition, such factors as pH and molarity of the covering film would be likely to have a significant impact on the dynamics of the soft tissue:salivary film fluoride exchange. What was clear, however, was that even after multiple applications of fluoride-free deionised water, detectable quantities of fluoride were released from the buccal mucosa up to two hours post fluoride application.

Easily detectable levels of fluoride in buccal mucosa were identified at the start of this study, in spite of the withdrawal of topical fluoride agents from the subjects. As this occurred in all five individuals, it seems unlikely to be attributable to protocol violations, and may be due to continued intake of fluoride *via* the diet, specifically from tea. Alternatively, it may be that two weeks is an insufficient length of time to allow complete clearance of fluoride from the oral mucosa, in individuals who have been applying topical fluoride products on a daily basis for ten to twenty years. The effect of withdrawing use of fluoride agents over a longer period is the topic of the final study in this chapter.

6.4.5 A Pilot Fluoride Withdrawal Study.

The aim of this study was to determine the effect of withdrawal and subsequent reintroduction of topical fluoride on the fluoride levels recorded in the buccal mucosa. Once again, five subjects were identified and provided with a non-fluoridated dentifrice to use for the first stage of the experiment. Baseline buccal mucosa and mixed saliva samples were collected from all the volunteers, who then started using the non-F dentifrice twice daily, and refrained from using any other fluoride-containing product. Samples of mucosa and saliva were collected twice weekly and stored, according to the protocol described earlier in this chapter. In view of volunteer unhappiness with prolonged periods on non-fluoridated pastes, a 1500 ppm NaF dentifrice was substituted for the non-F dentifrice after four weeks and samples collected over a further four week period. A plateau in salivary fluoride was established after four weeks on a non-fluoridated paste in the study in Chapter 4, and it was

anticipated therefore, that this was a sufficient time period.

Great care was taken to avoid any overlap of the sample sites (three sites on the right mucosa and three on the left), and on the advice of a consultant histopathologist it was deemed that a minimum of three weeks should elapse before the same site provided a repeat sample, to ensure adequate tissue maturation. The results of this study of dentifrice fluoride absorption are given in Table 6.4 and Figures 6.14 and 6.15.

The individual profiles are shown in Figure 6.14, and evidence a gradual decline in both salivary and mucosa fluoride levels over the first four weeks of the study for all except Subject 2. In this individual the mucosal levels remained fairly consistent until the last week on the non-F dentifrice (week 4), when they also began to decline. This individual also had consistently higher salivary fluoride levels than the remaining volunteers. The actual concentration of fluoride in saliva was much as would be expected on a non-F paste (see Table 6.4 and Chapter 4) and the baseline mucosa values were in a similar range to the studies described earlier in this chapter (mean 12.19 ppm). These mucosal levels fell to a mean of 3.69 ppm four weeks later, although there is a suggestion from the individual profiles that they would have declined still further given more time on the non-fluoridated dentifrice.

On the re-introduction of the 1500 ppm F dentifrice, all subjects showed an immediate increase in both salivary and mucosal fluoride levels, which rose steadily for the remainder of the experimental period in Subjects 1, 3 and 5, and also increased in Subjects 2 and 4, although in a less regular fashion.

In Figure 6.15, the mean (S.E.) fluoride concentration for all subjects (n=5) is plotted at each time point (note the log scale of the y-axis). The overall pattern is very similar for both parameters, although the saliva levels appear to plateau rather earlier than the mucosa levels. Interestingly, the final levels in both saliva and mucosa are slightly higher than the baseline

levels recorded at the outset of the experiment.

Table 6.4 **Mean (S.E.) mixed saliva and buccal mucosa fluoride concentrations with non-F and 1500 ppm F dentifrices for each subject (n=8 samples).**

	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5
Saliva: non-F	0.016 (0.002)	0.027 (0.002)	0.017 (0.002)	0.013 (0.002)	0.011 (0.001)
Saliva: 1500	0.034 (0.003)	0.031 (0.001)	0.033 (0.002)	0.034 (0.004)	0.032 (0.001)

Overall salivary mean:- *after 4 weeks on non-F dentifrice = 0.014 ppm*

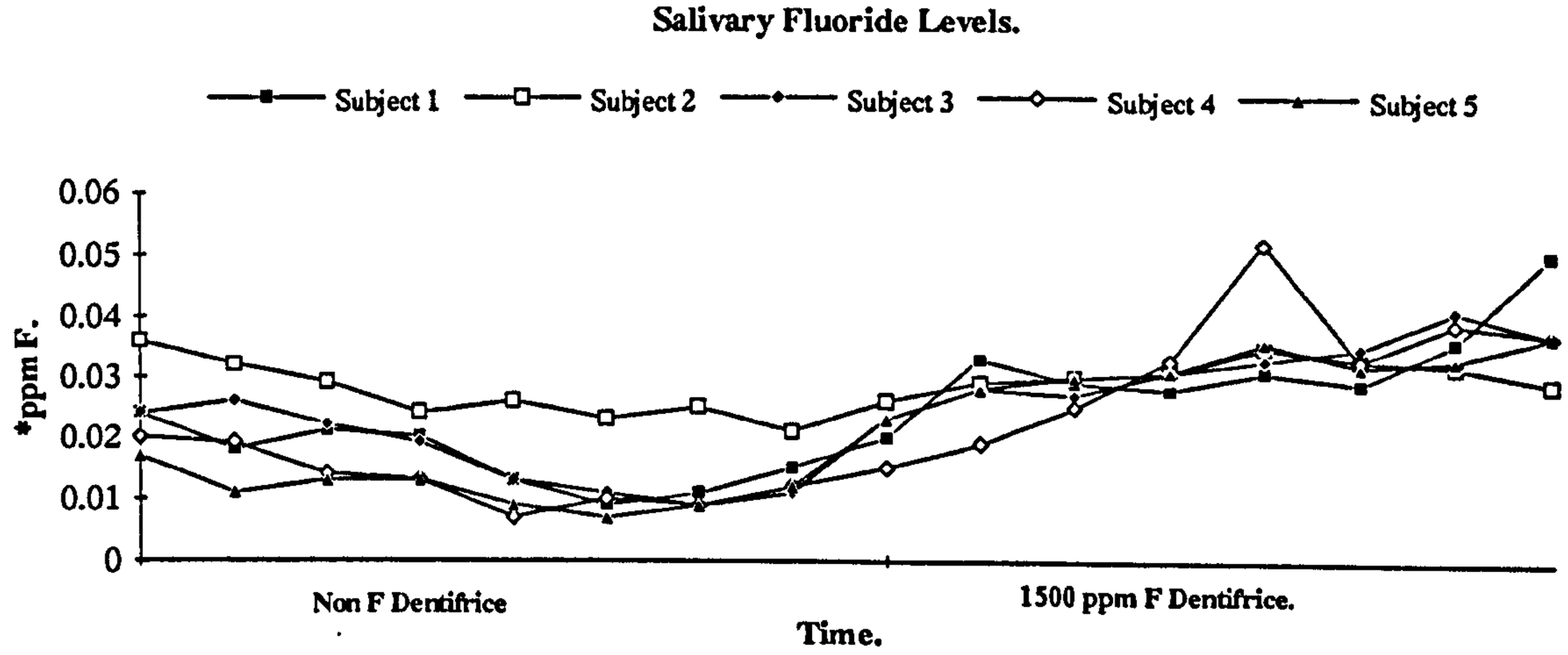
after 4 weeks on 1500 ppm dentifrice = 0.038 ppm

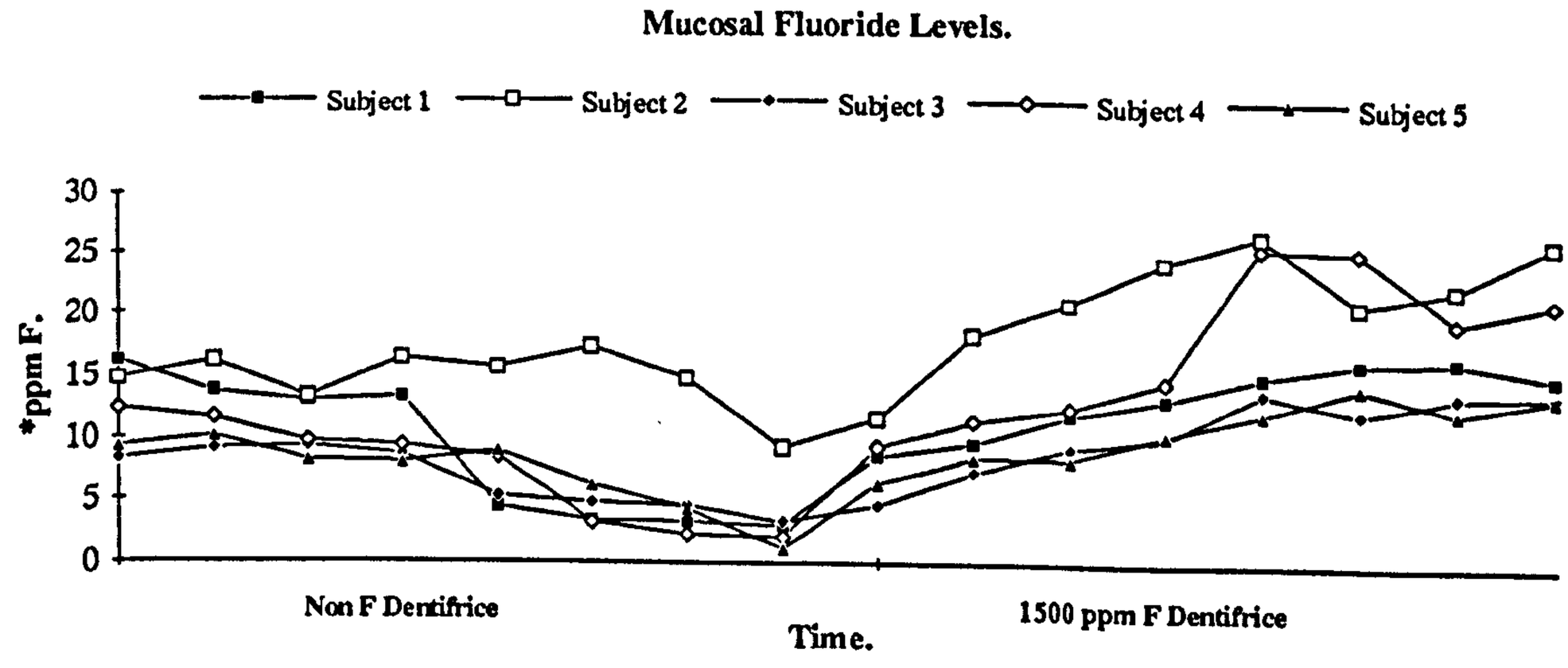
	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5
Mucosa: non-F	8.74 (2.02)	14.65 (0.89)	6.62 (0.86)	7.35 (1.49)	7.01 (1.07)
Mucosa:1500	13.89 (0.95)	22.78 (1.16)	11.41 (0.97)	18.59 (2.23)	11.35 (0.89)

Overall mucosal mean:- after 4 weeks on non-F dentifrice = 3.69 ppm

after 4 weeks on 1500 ppm dentifrice = 17.98 ppm

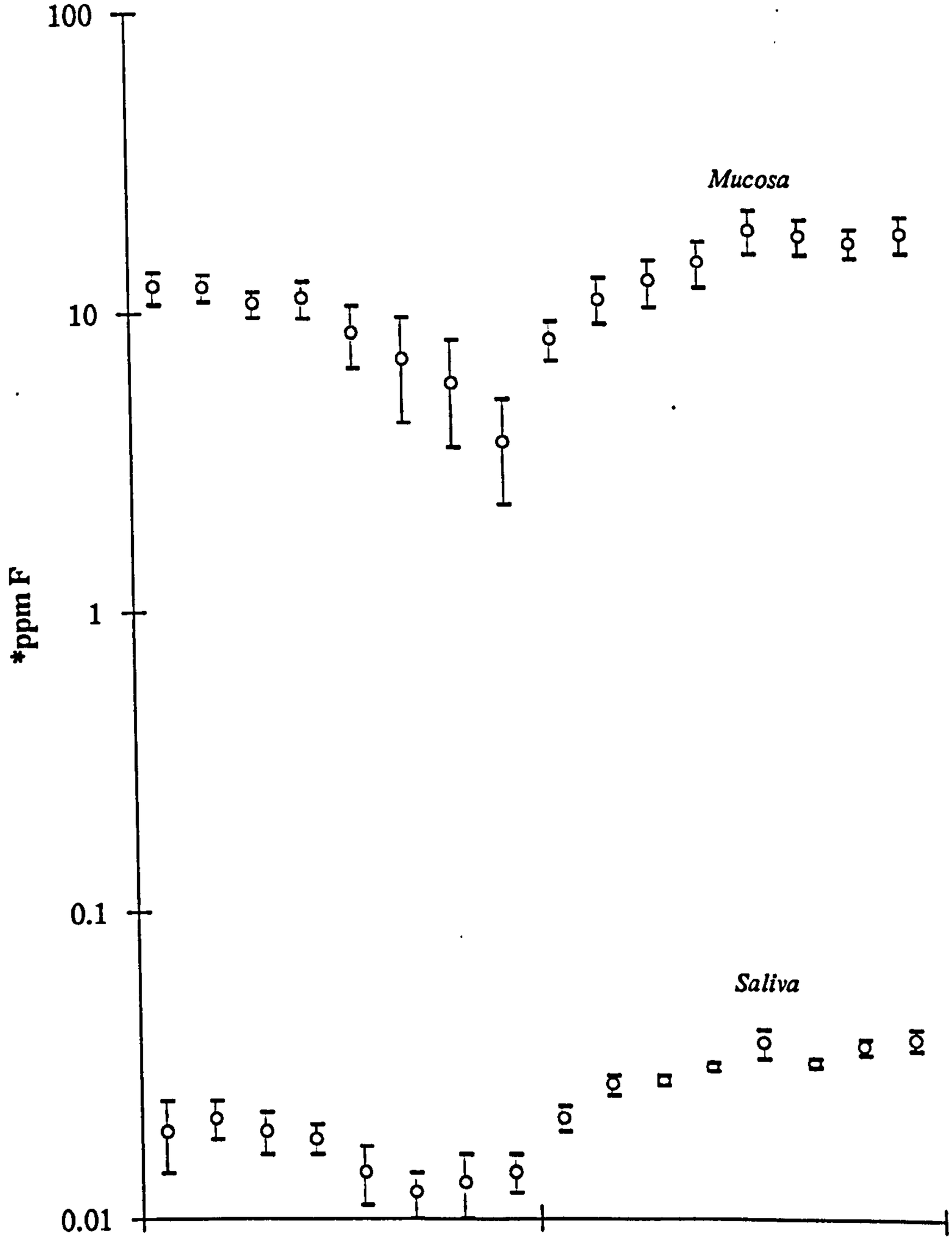
Figure 6.14 **Ambient mixed saliva and buccal mucosa fluoride levels with Non-F and 1500 ppm F dentifrices (5 subjects).**





*Note different y-axis scales.

Figure 6.15 Mean (S.E.) mixed saliva and buccal mucosa fluoride levels with non-F and 1500 ppm F dentifrices (n=5).



6.5 Discussion.

The clearance of substances from the oral cavity is a very complex phenomenon, particularly where a given substance is derived from both intra-oral and extra-oral sources, and can interact with the integuments of the mouth. The original concept of the mouth as a simple siphon is not sufficiently sophisticated to predict accurately the clearance time of substances such as fluoride, which do not undergo a straightforward process of dilution by saliva and subsequent swallowing.

The ability to predict intra-oral clearance half-lives would be of benefit, both for potentially therapeutic agents such as fluoride, and for potentially pathogenic agents such as sucrose and other fermentable carbohydrates. The mathematical modelling of Dawes (1983), Ekstrand *et al.* (1986) and Lagerlöf & Oliveby (1990), leave little doubt that fluoride is stored in the oral cavity following topical application. This "store" is likely to consist of a number of different fluoride sources, which are variously depleted and restocked, depending on the prevailing conditions. Undoubtedly, calcium fluoride is deposited on dental hard tissues after fluoride dentifrice, rinse or gel applications (Arends, Reintsema & Dijkman,1988; Dijkman & Arends,1988) and therapeutic claims have been made on its behalf (Ögaard, Rölla & ten Cate,1987). However, the dissolution rate of calcium fluoride in saliva is extremely slow (Rölla & Ögaard,1986; Saxegaard & Rölla,1988) and it seems unlikely, therefore, that this fluoride modality could have a significant impact on clearance in the hours immediately following topical application.

Plaque fluoride is also widely quoted as a potential source and contributor to salivary fluoride, particularly under conditions of reduced pH (Ophaug *et al.*1987). However, the major portion of fluoride in plaque also exists as calcium fluoride and is relatively insoluble in saliva (12-15 mg/L). In fact, calcium fluoride deposits have been observed persisting on tooth enamel for two weeks or more, after a single topical application of neutral 2% NaF.

Apart from sporadic appearances in the literature, very little attention has been directed towards identifying a role for the soft tissues in oral clearance. This seems to be something of an oversight, bearing in mind that approximately 80% of the total surface area of the mouth is covered with mucosa, and that it is known to be permeable to a wide range of substances.

The studies reported in this chapter constitute pilot investigations into the potential role of the oral mucosa as a factor in determining fluoride clearance rates and ambient salivary fluoride concentrations. In all the studies, significant levels of fluoride were recorded in the buccal mucosa at baseline, surely a reflection of fluoride adsorption to the oral soft tissues following repeated application? Furthermore, the pattern of fluoride clearance from the mucosa appears to follow a similar trend to that of mixed saliva, albeit at significantly higher levels, and possibly with a somewhat more prolonged half-life. Where studies have been undertaken which included use of products with a variety of fluoride concentrations, many workers have reported a so-called "carry-over" effect from one level of fluoride to the next (Schäfer,1989; Damato,1990). It may be that the intra-oral reservoirs of fluoride, including that within the superficial soft tissues, require a finite time to re-establish an equilibrium following the introduction of a new agent. Certainly, in the last study reported in this chapter, mucosal fluoride levels remained in the 3-4 ppm F range, even after four weeks on a non-fluoridated dentifrice.

In conclusion, it seems clear that the oral mucosa in general, and the buccal mucosa in particular, is responsible for the adsorption of significant quantities of fluoride from topical agents, and that this fluoride is available for release back into solution in saliva, should conditions become favourable. However, exactly what constitutes favourable conditions, and the nature of the interplay between saliva and mucosa are currently unknown.

Chapter 7. Discussion & Conclusions.

The original framework of this thesis was drafted in response to a series of recommendations published in 1990, following the International Symposium on Fluorides (Mechanisms of Action & Recommendations for Use). The list of recommendations was extensive and included the following :- 1). Use *in vitro* and intra-oral models to determine the optimum "low" fluoride concentrations and time needed to inhibit demineralisation completely, and/or effectively enhance remineralisation i.e. determine the "therapeutic window", 2). Define the reasons why whole saliva has increased levels of fluoride over duct saliva, and 3). Determine whether the mucosa can act as a reservoir of fluoride in the mouth.

The work reported here has attempted to address each of these issues.

7.1 Ambient Fluoride.

It became clear, during the early stages of this project, that the terminology surrounding fluoride kinetics was ambiguous. The term "baseline fluoride" is used in a variety of situations, e.g. it may be used to describe the level of fluoride at the start of an experiment, or it may be used to describe the background level of fluoride in saliva, plaque etc., in the absence of a recent topical or systemic fluoride dose. This second usage is inappropriate, as this particular "baseline" level is highly variable, and depends on many factors such as the fluoride concentration of agents used, salivary flow rates etc. In order to avoid confusion, the term "baseline fluoride" was restricted in this thesis to the first sample taken at, or before, the start of an experiment. The term "ambient fluoride" was used to describe the low-levels of fluoride found some hours after fluoride administration.

Ambient salivary fluoride levels are currently held in much greater respect than 10 years ago. Publication of *in vitro* studies showing cariostatic effects of sub-ppm fluoride concentrations

has resulted in increased interest in low levels of fluoride in saliva and plaque fluid, as the potential for the inhibition of demineralisation of enamel is recognised.

Concurrent with this recognition, commercial organisations responsible for the manufacture and distribution of fluoridated dentifrices, have introduced an almost empirically-derived range of products with reduced fluoride content, largely in response to concerns over an increased prevalence of fluorosis. However, many of these new products remain largely untested, particularly with respect to their long-term clinical efficacy. Their claims of effectiveness are supported either by data extrapolated from higher fluoride dentifrices, often tested on an inappropriate age-group of children, or by studies with inadequate controls. Indeed, as recently as the July 1995 issue of the "B.D.A. news", these problems were again highlighted.

The clinical studies on ambient fluoride reported in this thesis, demonstrate clearly that twice daily application of a lower concentration fluoride dentifrice results in reduced fluoride levels in saliva, plaque and plasma, when tested in adults. Whilst it is difficult to isolate one intra-oral parameter as the most significant in determining caries progression, it seems very likely that the fluoride concentration at the enamel:solution interface, the biophase, is one of the most important factors. This fluoride concentration results from the contributions of saliva, plaque fluid, plasma *via* the gingival crevicular fluid, and the superficial enamel. Therefore any changes which affects these parameters will surely have an indirect effect on the biophase.

There is considerable pressure, particularly in areas where caries-rates are low, for parents and children to be encouraged to use dentifrices containing less than 1000 ppm fluoride. It is vitally important, before widespread use of these products is endorsed, that further *in vitro* and *in situ* testing is instigated, and definitive, double-blind clinical studies are undertaken. This is particularly crucial for those children living in areas where caries-rates are increasing.

The *in vitro* study reported here, attempted to duplicate the effects of the range of ambient salivary fluoride levels found in the low fluoride dentifrice clinical study. In general, the results indicated that even very low levels of fluoride (0.02 ppm) were sufficient to reduce demineralisation. However, the only net remineralisation in this experiment was recorded for ΔZ, with 0.06 ppm fluoride in solution. The relative merits of demineralisation inhibition and remineralisation enhancement are unclear. Clearly, in a "caries-free" child, the frequent application of low concentrations of fluoride may be sufficient to completely inhibit enamel demineralisation, and perpetuate their caries-free status. For the majority of the population, however, this is unlikely to be sufficient, and active remineralisation of early lesions is necessary, requiring conditions more in favour of mineral uptake and deposition, including higher levels of available fluoride.

As always, it is dangerous to over-interpret *in vitro* data. The artificial nature of the experiment, the pH-cycling times, the re-/demineralising solutions and the carious lesions themselves, all differentiate this study from the *in vivo* situation. Increasing the sensitivity of *in vitro* techniques, including analytical methods such as laser fluorescence, is vital if pH-cycling studies are to remain significant contributors to understanding in this field.

7.2 Fluoride Clearance.

The peak concentrations of fluoride found immediately following topical fluoride application, and the ambient levels found hours later, are not separate entities, but are intimately related. The ambient level of fluoride at any given time is dependent on the pattern of fluoride intake and ingestion prior to that time, and will be increased following repeated administration. It is generally accepted that peak concentrations of fluoride are probably not critical, *per se,* but result in the deposition of large quantities of fluoride in the oral cavity, a proportion of which is available for periods afterwards.

The prolonged nature of intra-oral fluoride clearance is established. The deposition of CaF_2

on enamel and in plaque fluid may well contribute to fluoride in saliva, but given this material's relative insolubility, it seems likely that its contribution is to maintain ambient fluoride levels, rather than in the 1-2 hour period immediately post-application.

There seems little doubt that the oral soft tissues adsorb/absorb significant quantities of fluoride. Currently, the nature of this process is not known, but it may be that it is facilitated by a reduced pH. If fluoride is taken up as neutral HF, it would be readily available, -much more so than insoluble CaF_2- to be released back into solution when the concentration gradient and local conditions were favourable. Future studies on soft tissue uptake using fluoride solutions of different pH values would help answer this question.

7.3 Conclusions.

During the course of this thesis, efforts were made to establish the effect of reduced dentifrice fluoride concentrations on ambient levels of fluoride, primarily in whole saliva, but also in plasma and dental plaque. In addition, the influence of these ambient fluoride levels on the balance between enamel remineralisation and demineralisation were investigated using an *in vitro* model, and attempts were made to establish the role of the oral soft tissues in maintaining intra-oral fluoride levels following topical fluoride administration.

As a result, it would seem the fluoride concentration of sodium fluoride dentifrices has a significant effect on the ambient fluoride levels in saliva, plasma and plaque, and further investigations are required to ensure that reducing the fluoride content of dentifrices will not be to the detriment of their anti-caries efficacy. Furthermore, fluoride from sodium fluoride dentifrices and mouth rinses is absorbed by the buccal mucosa, and may contribute in a beneficial manner to the prolonged intra-oral clearance time of the fluoride ion.

Appendix 1. Approval letter for "Investigations relating to Clinical Topical Fluoride Therapies" from Area Dental Ethics Committee.

GREATER GLASGOW HEALTH BOARD

225 BATH STREET
GLASGOW G2 4JT
Telephone: 041-204 2755

Our Ref.: RMcK/EMcG

Your Ref.:

If 'phoning ask for:— Mr R. McKechnie

All communications must be addressed to .....

10 May 1985

Professor K.W. Stephen
Department of Oral Medicine and Pathology
Glasgow Dental Hospital and School
378 Sauchiehall Street
GLASGOW
G2 3JZ

Dear Professor Stephen

AREA DENTAL ETHICS COMMITTEE

I write to inform you that your application of 6th August 1984 for clinical research has now been approved by the Area Dental Ethics Committee at their meeting on 11th March 1985, the aim of the project being:

"Investigations Relating to Clinical Topical Fluoride Therapies"

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

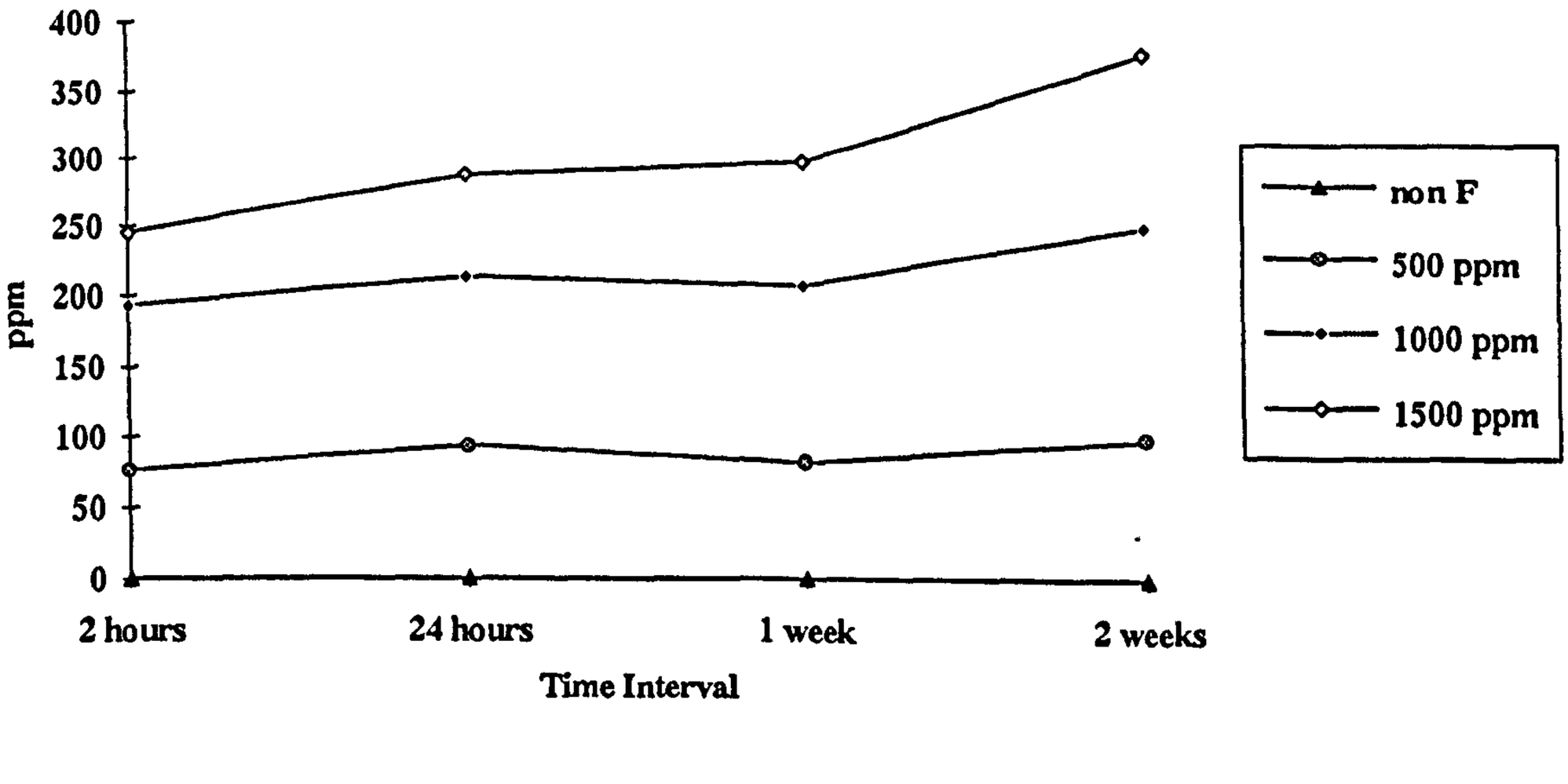
R. McKECHNIE
Chief Administrative Dental Officer

Copy for infor. Mr H A Critchlow, Chairman
Mr G Lewis, Secretary

Appendix 3.1 Test Dentifrice Fluoride Concentrations.

	Dentifrice Fluoride Concentration			
Time Interval	**non F**	**500 ppm F**	**1000 ppm F**	**1500 ppm F**
Mean (S.D.)	0.22 (0.008)	87 (9.7)	215 (24.5)	301.25 (55)
2 hours	0.23	76	193	245
24 hours	0.21	93	212	287
1 week	0.22	82	206	297
2 weeks	0.22	97	250	376

Ionized Fluoride in 20% Aqueous Slurries of NaF Dentifrices over Time.



Appendix 3.2 Volunteer Protocol

Thank you for agreeing to participate in this dentifrice study.

1. You will be provided with toothpaste of varying fluoride concentrations, and with toothbrushes which you should use for the duration of the experiment.

2. Please use the dentifrice twice daily, morning and night. If you wish to brush your teeth more often than this, at lunchtime for example, you will be provided with a non-F dentifrice for this purpose.

3. Do not use any other fluoride containing product eg another dentifrice or mouthrinse, for the duration of the study.

4. It is important that volunteers use roughly equivalent amounts of toothpaste (approx. ½ brush length). The appropriate amount will be demonstrated at the start of the study.

5. You will be asked to complete a questionnaire in relation to your toothbrushing habits.

6. Unstimulated saliva samples will be collected at 12 noon every 2nd week day throughout the experimental period.

7. Each dentifrice will be used for a period of 4 weeks, making a total experimental time of 16 weeks. You will not know which dentifrice you are using.

8. Please do not rinse your mouth after toothbrushing. Rinsing with a glass of water, for example, will wash much of the fluoride out of your mouth, and may affect the experimental results.

Appendix 3.3 Protocol for collection of unstimulated saliva samples for fluoride analysis.

1. Toothbrushing should be completed 4 hours prior to saliva sampling.

2. No tea should be consumed for at least 1 hour prior to saliva collection.

3. To minimise psychic stimulation, the subject should not be aware of the exact timing of the sample collection.

4. Where a sample volume of 0.5 ml is sufficient, the subject should simply void all saliva present in the mouth into a plastic universal vial.

5. Where a greater sample volume is required the subject should void all the saliva in the mouth prior to starting collection. Then, he should sit comfortably, with the head slightly forward. Every 30 seconds, over a 5 minute period, all the saliva in the mouth should be voided into the collecting tube.

6. The movements of the oral musculature should be kept to a minimum. Mouth-breathing should be avoided.

7. When sufficient weight/volume has been collected, the sample should either be analysed immediately, or sealed and stored at -16°C until required.

Appendix 3.4 Protocol for measuring low-levels (<1 ppm) F using *Orion 901 Ionalyser.*

This method is suitable for measuring fluoride levels in the range 0.01-1.0 ppm. At levels below 0.4ppm, low-level TISAB and the SET BLANK facility on the *901 Ionalyser* must be used.

1. Equipment and solutions:

901 Ionalyser (*Orion Research Incorporated Ltd., Mass., U.S.A.*)

Fluoride specific electrode *(Orion* 96-09)

Single junction (Ag/AgCl) reference electrode (*Orion* 90-01)

Magnetic Stirrer

Plastic labware

Equitransferrent filling solution (*Orion Research*)

Standard fluoride (NaF) solutions; 0.1M (940906)

100ppm (940907)

Total Ionic Strength Adjustor (TISAB II, III or low-level)

Distilled, de-ionised water.

Microsample dishes (*Orion Research)*

2. Method:

Electrode function is checked by establishing the slope of the mV response to changes in fluoride concentration. This slope must be established using concentrations of fluoride which lie within the linear portion of the concentration : mV curve.

Therefore, before measuring standards or samples, the electrodes must be checked to ensure they are functioning correctly by the following procedure:

Place 10mls of deionised water and 10mls of low-level TISAB into a plastic beaker. Immerse the electrodes in the solution and ensure the sensing element of both are completely covered. Thereafter, turn the thumbwheel of the 901 meter to 'REL mV mode'. At this point, only the CLEAR/read mV button should be lit, and the digital readout obtained is in absolute millivolts.

Via a micropipette, 100 µls of 1ppm standard (0.1 ppm) is then added and stirred thoroughly. When a stable reading is obtained, or after a predesignated time, the electrode potential in recorded in millivolts.

Press CLEAR/READ mV. Press SET CONC, at which time the digital display should read 0.000.

1 ml of the same standard solution (1ppm) is then pipetted into the plastic beaker and stirred thoroughly. The digital display should then stabilise in the range 54-60 mV. This is a reading in relative millivolts i.e. the electrode system should record a 54-60 mV difference for a 'times 10' change in F concentration.

This value is entered onto the thumbwheel switches labelled 'SLOPE'. The electrodes are removed from the solution, rinsed with deionised water and blotted dry.

The system is now ready for calibration.

3. Calibration.

Two options are available for calibration : the Direct method, where the meter is set to give readings in concentration units, and the indirect method, where the meter is used in the RELmV mode, and the millivolt readings obtained are then plotted manually, or using a computer programme (FLUOFIT) to convert to concentration. The Direct method is described first:

(a). The method described is suitable for calibrating the 901 meter when the CONC mode is used and the digital display is a direct reading of sample concentration.

The thumbwheel switch is turned to CONC mode and the electrodes are placed into 1ml of 1 ppm F/TISAB stock solution (using *Orion* microsample dishes facilitates the measurement of small sample volumes). It is critical that the solution used at this stage is accurate in its concentration as all subsequent concentrations will be calculated using this as a reference.

A measurement time is selected i.e. the time after the electrodes are sumberged in the sample when the reading will be taken e.g. 5 min. This time should remain the same for any group of samples which are to be compared. For low levels, 10 min per sample should be used. Once the reading is stable the CLEAR/READ mV button, is pressed, which will light at this stage. The thumbwheels for 'Standard' must read the appropriate concentration, in this instance 001.00. The SET CONC button is now pressed. The readout changes from approximately 104-106 mV (absolute mV for 1ppm) to 001.00, the concentration of the standard. If required, another standard can be measured at this stage to check the electrode slope.

Before any samples containing low levels of fluoride are measured, it is necessary to activate the SET BLANK facility on the machine. The purpose of this is to eliminate from any sample measurements the small amount of fluoride which may be present in either deionised water or the TISAB. However, this is only of significance where the levels of fluoride are below 0.4 ppm. To engage the SET BLANK facility the electrodes are removed from the standard solution and rinsed with deionised water. Subsequently, they are placed in 0.5 ml of deionised water and 0.5 ml low level TISAB in a microsample dish.The system is then left to stabilise for the prescribed time. The value for the SET BLANK solution must be <0.006 ppm. The SET BLANK button is then pressed, and will now light along with the SET CONC button. The CLEAR/READ mV button will be extinguished. The digital display should read 000.00.

The meter/electrode system is now calibrated to read within the range 1.00 - 0.01 ppm. In addition, standards must be inserted between the samples during extended measuring times to ensure the continued accurate functioning of the system.

Furthermore, recalibration should be carried out every 3 hours.

(b). The Indirect method: this technique simply records the mV reading for each of 5-6 standard solutions eg. 0.01, 0.02, 0.05, 0.1, 0.5, 1.0 ppm, when added to equal volumes of TISAB, with the meter set in the

RELmV mode. The standards should be measured in order of increasing concentration, to minimise the effect any fluoride carryover from one standard to the next. The electrodes should be thoroughly rinsed in de-ionised water and blotted dry between each measurement. The test samples are then introduced, following the same timing and cleaning procedures as detailed above. The mV readings are recorded, and plotted subsequently aginst the calibration curve drawn from the standard solution mV readings, or alternatively entered into a software programme (FLUOFIT), which fits a curve to the standards, and calculates the corresponding concentrations from the mV readings of the samples. This technique is more time-consuming, but is free of the disadvantage of being dependent to a great extent on the accuracy of the concentration of one standard solution, which is inherent in the Direct technique.

Appendix 3.5 Salivary Fluoride Concentrations : Raw data relating to Chapter 3

	Volunteer 1		2		3		4	
Time (Days)	1st Dentifrice		1st Dentifrice		1st Dentifrice		1st Dentifrice	
0	1500 ppm F:	0.045	500 ppm F:	0.012	1000 ppm F:	0.018	500 ppm F:	0.021
2		0.042		0.011		0.019		0.020
4		0.040		0.012		0.020		0.021
7		0.041		0.016		0.019		0.023
9		0.041		0.012		0.022		0.025
11		0.041		0.014		0.023		0.027
14		0.042		0.009		0.023		0.027
16		0.041		0.009		0.024		0.026
18		0.040		0.010		0.023		0.025
21		0.041		0.011		0.028		0.024
23		0.038		0.010		0.022		0.027
25		0.039		0.011		0.021		0.029
28		0.039		0.009		0.028		0.029
30		0.040		0.010		0.026		0.030
	2nd Dentifrice		2nd Dentifrice		2nd Dentifrice		2nd Dentifrice	
0	500ppm F:	0.038	1500 ppmF:	0.013	non F:	0.029	1500 ppm F:	0.030
2		0.029		0.013		0.019		0.035
4		0.014		0.016		0.017		0.029
7		0.014		0.019		0.016		0.030
9		0.015		0.021		0.016		0.031
11		0.015		0.022		0.016		0.031
14		0.014		0.022		0.015		0.030
16		0.014		0.022		0.015		0.029
18		0.013		0.021		0.014		0.029
21		0.013		0.022		0.013		0.031
23		0.013		0.023		0.013		0.032
25		0.012		0.020		0.011		0.034
28		0.012		0.022		0.012		0.033
30		0.012		0.022		0.011		0.033

	Volunteer 1		2		3		4	
Time (Days)	3rd Dentifrice		3rd Dentifrice		3rd Dentifrice		3rd Dentifrice	
0	non F:	0.012	non F:	0.022	1000 ppm F:	0.012	non F:	0.032
2		0.012		0.021		0.002		0.034
4		0.013		0.017		0.022		0.032
7		0.010		0.010		0.021		0.021
9		0.008		0.008		0.019		0.040
11		0.005		0.007		0.002		0.018
14		0.006		0.007		0.018		0.010
16		0.006		0.007		0.017		0.014
18		0.007		0.008		0.018		0.010
21		0.008		0.008		0.018		0.009
23		0.009		0.009		0.019		0.011
25		0.007		0.009		0.023		0.010
28		0.006		0.008		0.024		0.012
30		0.007		0.010		0.025		0.012
	4th Dentifrice		4th Dentifrice		4th Dentifrice		4th Dentifrice	
0	1000 ppm F:	0.006	Incomplete:	*	500 ppm F:	0.025	1000 ppm F:	0.030
2		0.009		*		0.020		0.022
4		0.010		*		0.019		0.025
7		0.010		*		0.020		0.021
9		0.015		*		0.019		0.020
11		0.012		*		0.018		0.030
14		0.013		*		0.016		0.028
16		0.013		*		0.019		0.021
18		0.013		*		0.019		0.022
21		0.014		*		0.018		0.023
23		0.015		*		0.022		0.023
25		0.016		*		0.021		0.026
28		0.020		*		0.020		0.025
30		0.018		*		0.019		0.026

	Volunteer 5		6		7		8	
Time (Days)	1st Dentifrice		1st Dentifrice		1st Dentifrice		1st Dentifrice	
0	1500 ppm F:	0.018	non F:	0.015	non F:	0.008	500 ppm F:	0.026
2		0.018		0.014		0.010		0.025
4		0.020		0.013		0.010		0.021
7		0.019		0.011		0.012		0.023
9		0.020		0.013		0.013		0.022
11		0.020		0.014		0.012		0.018
14		0.018		0.013		0.008		0.022
16		0.019		0.012		0.008		0.019
18		0.021		0.014		0.007		0.022
21		0.020		0.012		0.006		0.031
23		0.022		0.013		0.007		0.026
25		0.024		0.014		0.006		0.024
28		0.029		0.013		0.006		0.026
30		0.029		0.012		0.006		0.027
	2nd Dentifrice		2nd Dentifrice		2nd Dentifrice		2nd Dentifrice	
0	500 ppm F:	0.031	1000 ppmF:	0.012	1000 ppmF:	0.007	1500 ppmF:	0.030
2		0.030		0.011		0.020		0.029
4		0.010		0.012		0.012		0.029
7		0.014		0.020		0.013		0.028
9		0.010		0.040		0.014		0.029
11		0.009		0.028		0.015		0.030
14		0.009		0.040		0.014		0.026
16		0.010		0.027		0.013		0.030
18		0.010		0.022		0.014		0.031
21		0.023		0.020		0.013		0.031
23		0.018		0.019		0.014		0.031
25		0.010		0.019		0.017		0.032
28		0.022		0.018		0.015		0.030
30		0.019		0.019		0.014		0.029

	Volunteer 5	6	7	8
Time (Days)	3rd Dentifrice	3rd Dentifrice	3rd Dentifrice	3rd Dentifrice
0	non F: 0.011	1500 ppm F: 0.019	1500 ppmF: 0.013	non F: 0.03
2	0.017	0.028	0.012	0.032
4	0.007	0.032	0.042	0.03
7	0.007	0.028	0.025	0.028
9	0.008	0.025	0.013	0.028
11	0.020	0.020	0.020	0.022
14	0.010	0.021	0.013	0.025
16	0.009	0.022	0.013	0.027
18	0.007	0.022	0.014	0.019
21	0.007	0.024	0.013	0.022
23	0.005	0.025	0.014	0.024
25	0.005	0.024	0.015	0.019
28	0.004	0.025	0.016	0.020
30	0.004	0.024	0.015	0.017
	4th Dentifrice	4th Dentifrice	4th Dentifrice	4th Dentifrice
	1000 ppm F	500 ppm F:	500 ppm F:	1000 ppm :
0	0.005	0.025	0.014	0.022
2	0.007	0.026	0.013	0.021
4	0.001	0.025	0.013	0.023
7	0.015	0.024	0.014	0.022
9	0.017	0.025	0.013	0.026
11	0.016	0.025	0.011	0.024
14	0.017	0.024	0.009	0.027
16	0.018	0.022	0.009	0.025
18	0.023	0.022	0.008	0.025
21	0.021	0.018	0.008	0.026
23	0.022	0.017	0.007	0.025
25	0.022	0.015	0.007	0.027
28	0.021	0.015	0.005	0.027
30	0.022	0.014	0.007	0.026

	Volunteer 9		10		11		12	
Time (Days)	1st Dentifrice		1st Dentifrice		1st Dentifrice		1st Dentifrice	
0	1500 ppm F:	0.028	1000 ppm F	0.040	1000 ppm F	0.032	1000 ppm F:	0.022
2		0.027		0.039		0.030		0.023
4		0.027		0.035		0.032		0.023
7		0.027		0.034		0.035		0.025
9		0.025		0.032		0.037		0.023
11		0.022		0.034		0.032		0.025
14		0.025		0.035		0.033		0.022
16		0.023		0.034		0.035		0.021
18		0.025		0.034		0.035		0.020
21		0.022		0.038		0.037		0.022
23		0.021		0.036		0.036		0.023
25		0.023		0.023		0.037		0.023
28		0.030		0.038		0.038		0.024
30		0.026		0.042		0.037		0.025
	2nd Dentifrice		2nd Dentifrice		2nd Dentifrice		2nd Dentifrice	
0	500 ppm F:	0.027	non F:	*	non F:	0.042	non F:	0.027
2		0.015		0.032		0.019		0.010
4		0.015		0.030		0.013		0.009
7		0.015		0.030		0.013		0.009
9		0.015		0.029		0.012		0.009
11		0.017		0.029		0.012		0.009
14		0.015		0.028		0.011		0.009
16		0.014		0.027		0.011		0.010
18		0.017		0.026		0.012		0.009
21		0.024		0.026		0.011		0.009
23		0.015		0.025		0.011		0.010
25		0.022		0.023		0.011		0.009
28		0.017		0.022		0.010		0.009
30		0.015		0.021		0.010		0.010

	Volunteer 9	10	11	12
Time (Days)	3rd Dentifrice	3rd Dentifrice	3rd Dentifrice	3rd Dentifrice
0	non F: 0.014	1000 ppmF: 0.022	1000 ppmF: 0.010	1000 ppm F: 0.018
2	0.012	0.025	0.022	0.032
4	0.010	0.037	0.031	0.031
7	0.008	0.042	0.032	0.028
9	0.008	0.041	0.032	0.026
11	0.008	0.040	0.034	0.025
14	0.007	0.039	0.033	0.024
16	0.005	0.040	0.030	0.023
18	0.004	0.041	0.028	0.023
21	0.005	0.039	0.031	0.023
23	0.005	0.037	0.033	0.023
25	0.006	0.041	0.031	0.022
28	0.006	0.042	0.026	0.023
30	0.005	0.040	0.028	0.022
	4th Dentifrice	4th Dentifrice	4th Dentifrice	4th Dentifrice
0	1000 ppm F: 0.007	Incomplete: *	1500 ppm F 0.033	1500 ppm F: 0.023
2	0.028	*	0.032	0.023
4	0.014	*	0.026	0.024
7	0.038	*	0.027	0.025
9	0.026	*	0.036	0.026
11	0.036	*	0.032	0.025
14	0.034	*	0.033	0.025
16	0.031	*	0.035	0.026
18	0.029	*	0.034	0.027
21	0.026	*	0.035	0.027
23	0.021	*	0.039	0.028
25	0.022	*	0.037	0.030
28	0.020	*	0.033	0.029
30	0.024	*	0.037	0.031

Appendix 3.6 Tooth-brushing Questionnaire.

Please answer questions listed below as accurately as possible. The aim of the questionnaire is to determine the frequency and duration of your *normal* tooth-brushing habits.

1. How often, during a 24 hour period do you brush your teeth? _____

2. Give an estimate, in seconds and minutes, how long you spend each time you clean your teeth. _____

3. What brand of toothpaste do you routinely use? _____

4. Give an estimate of how much toothpaste, in centimetres, you use each time you clean your teeth e.g. ½ cm, 1½ cm etc. _____

5. When cleaning your teeth do you use only horizontal strokes, or do you include some vertical strokes as well? _____

6. Do you normally rinse your mouth with water when you clean your teeth? If so, how many times? _____

7. Do you spit out some toothpaste whilst you are brushing? _____

8. Do you routinely use any other method of plaque removal e.g. dental floss, dental tape woodsticks etc.? _____

9. How many cups of tea do you drink each day? What brand of tea do you use at home?

_____

Appendix 4.1 Salivary Fluoride Concentrations: Raw data relating to Chapter 4

	Volunteer 1	2	3	4
	Non F Dentifrice	Non F Dentifrice	Non F Dentifrice	Non F Dentifrice
0	0.013	0.013	0.020	0.008
1	0.003	0.006	0.003	0.001
2	0.003	0.008	0.001	0.002
3	0.005	0.012	0.008	0.012
4	0.007	0.005	0.003	0.009
5	0.011	0.012	0.012	0.004
6	0.013	0.027	0.012	0.005
7	0.007	0.009	0.009	0.006
8	0.012	0.016	0.010	0.005
9	0.012	0.015	0.008	0.004
10	0.011	0.008	0.003	0.005
11	0.016	0.015	0.009	0.007
12	0.015	0.021	0.012	0.010
	500 ppm F Dentifrice	500 ppm F Dentifrice	500 ppm F Dentifrice	500 ppm F Dentifrice
1	0.011	0.037	0.011	0.010
2	0.008	0.008	*	0.003
3	0.006	0.012	0.009	0.004
4	0.016	*	0.007	0.001
5	0.008	0.016	0.008	0.006
6	0.015	0.015	0.007	0.008
7	0.012	*	0.007	0.009
8	0.015	*	0.006	0.009
9	0.025	0.041	0.018	0.014
10	0.025	0.060	0.027	0.012
11	0.023	0.016	0.018	0.013
12	0.025	0.017	0.011	0.015

	Volunteer 1	2	3	4
	1000ppm F Dentifrice	1000 ppm F Dentifrice	1000 ppm F Dentifrice	1000ppm F Dentifrice
1	0.018	0.017	*	0.027
2	0.023	0.019	0.019	0.014
3	0.021	0.047	0.016	0.011
4	0.038	0.026	0.020	0.015
5	0.014	0.018	0.014	0.009
6	0.028	*	0.018	0.007
7	0.030	0.017	0.029	0.009
8	0.028	0.014	*	0.007
9	0.016	0.025	0.018	0.007
10	0.027	0.026	0.013	0.007
11	0.017	0.022	0.018	0.008
12	0.019	0.024	0.016	0.008
	1500ppm F Dentifrice	1500 ppm F Dentifrice	1500 ppm F Dentifrice	1500ppm F Dentifrice
1	0.023	0.059	0.024	0.031
2	0.032	0.025	0.018	0.026
3	0.036	0.027	0.016	0.021
4	0.038	0.029	0.020	0.019
5	0.035	0.031	0.022	0.017
6	0.028	0.029	0.024	0.018
7	0.031	0.026	0.018	0.019
8	0.027	0.028	0.019	0.021
9	0.030	0.032	0.022	0.018
10	0.038	0.041	0.021	0.019
11	0.036	0.038	0.019	0.016
12	0.042	*	0.043	0.019

	Volunteer 5	6	7	8
	Non F Dentifrice	Non F Dentifrice	Non F Dentifrice	Non F Dentifrice
0	0.01	0.013	0.011	0.012
1	0.012	0.010	0.007	0.006
2	0.002	0.001	0.020	0.001
3	0.010	0.005	0.018	0.007
4	0.015	0.007	0.006	0.003
5	0.009	0.014	*	0.007
6	0.013	0.017	0.014	*
7	0.006	0.014	*	0.007
8	0.014	0.017	0.015	0.007
9	0.013	0.015	0.011	0.006
10	0.012	0.018	0.013	0.002
11	0.011	0.015	0.012	0.006
12	0.020	0.017	0.020	0.019
	500 ppm F Dentifrice	500 ppm F Dentifrice	500 ppm F Dentifrice	500 ppm F Dentifrice
1	0.014	0.012	0.021	0.019
2	0.007	*	*	0.006
3	0.013	0.011	0.016	0.010
4	0.007	0.009	0.028	*
5	0.014	0.012	0.026	0.011
6	0.025	0.013	0.021	0.016
7	0.013	0.016	0.014	0.009
8	0.013	0.017	0.016	*
9	0.029	0.023	0.032	0.018
10	0.025	0.018	0.029	0.019
11	0.029	0.019	0.020	0.016
12	0.020	0.012	0.009	0.012

	Volunteer 5	6	7	8
	1000 ppm F Dentifrice	1000ppm F Dentifrice	1000 ppm F Dentifrice	1000 ppm F Dentifrice
1	0.026	0.021	0.030	0.020
2	0.020	0.024	0.034	0.021
3	0.020	0.031	0.052	0.026
4	0.025	0.018	*	0.023
5	0.025	0.019	0.022	0.012
6	0.015	0.016	0.028	0.017
7	0.013	0.013	*	0.022
8	0.016	*	*	0.022
9	0.012	0.011	0.040	0.017
10	0.014	0.011	0.042	0.013
11	0.015	0.020	0.031	0.015
12	0.018	0.017	*	0.016
	1500 ppm F Dentifrice	1500ppm F Dentifrice	1500 ppm F Dentifrice	1500 ppm F Dentifrice
1	0.026	0.043	*	0.026
2	0.027	0.027	*	0.017
3	0.029	0.023	0.042	0.019
4	0.022	0.031	0.039	0.022
5	0.019	0.030	0.036	0.021
6	0.022	0.025	0.037	0.019
7	0.024	0.027	0.041	0.024
8	0.026	0.021	0.032	0.021
9	0.025	0.032	0.035	0.023
10	0.021	0.028	0.031	0.029
11	0.023	0.026	0.032	0.026
12	0.048	0.053	0.027	0.031

	Volunteer 9	10	11	12
	Non F Dentifrice	Non F Dentifrice	Non F Dentifrice	Non F Dentifrice
0	0.016	0.011	0.007	0.010
1	0.014	0.008	0.010	0.010
2	0.013	0.005	0.001	0.001
3	0.009	0.010	0.006	0.010
4	0.003	0.028	0.008	0.020
5	0.009	0.016	0.007	0.009
6	0.020	0.017	0.015	0.019
7	0.009	0.016	0.007	0.009
8	0.010	0.013	0.009	0.012
9	0.014	0.018	0.012	0.013
10	0.009	0.005	0.005	0.003
11	0.010	0.014	0.009	0.014
12	0.011	0.010	0.011	0.012
	500 ppm F Dentifrice	500 ppm F Dentifrice	500 ppm F Dentifrice	500 ppm F Dentifrice
1	0.009	0.019	0.008	0.013
2	0.008	0.010	0.011	0.017
3	0.006	0.019	0.013	0.013
4	0.013	0.018	0.013	0.015
5	0.008	0.042	0.017	0.021
6	0.008	0.044	0.013	0.017
7	0.010	0.038	0.013	0.012
8	*	0.017	0.005	0.016
9	0.016	0.035	0.016	0.020
10	0.023	0.024	0.025	0.029
11	0.010	0.016	0.009	0.017
12	0.009	0.022	0.009	0.011

	Volunteer 9	10	11	12
	1000 ppm F Dentifrice	1000ppm F Dentifrice	1000 ppm F Dentifrice	1000 ppm F Dentifrice
1	0.017	0.047	*	0.030
2	0.020	0.037	*	0.038
3	0.016	0.017	0.009	0.014
4	0.029	0.017	0.015	0.0.20
5	0.013	*	0.015	0.015
6	0.011	*	0.016	0.013
7	0.017	0.045	0.013	0.022
8	0.010	0.041	0.012	0.038
9	0.013	0.044	*	0.021
10	*	0.025	0.019	0.024
11	0.012	0.026	0.018	0.030
12	0.014	0.022	0.019	0.030
	1500 ppm F Dentifrice	1500 ppmF Dentifrice	1500 ppm F Dentifrice	1500 ppm F Dentifrice
1	*	*	0.042	0.107
2	*	0.170	0.036	0.073
3	0.016	0.091	0.032	0.046
4	0.011	0.086	0.031	0.038
5	0.017	0.091	0.026	0.037
6	0.019	0.083	0.036	0.041
7	0.021	0.089	0.031	0.043
8	0.015	0.096	0.034	0.047
9	0.016	0.010	0.029	0.040
10	0.019	0.099	0.037	0.042
11	0.020	0.092	0.039	0.039
12	0.021	0.11	0.038	0.046

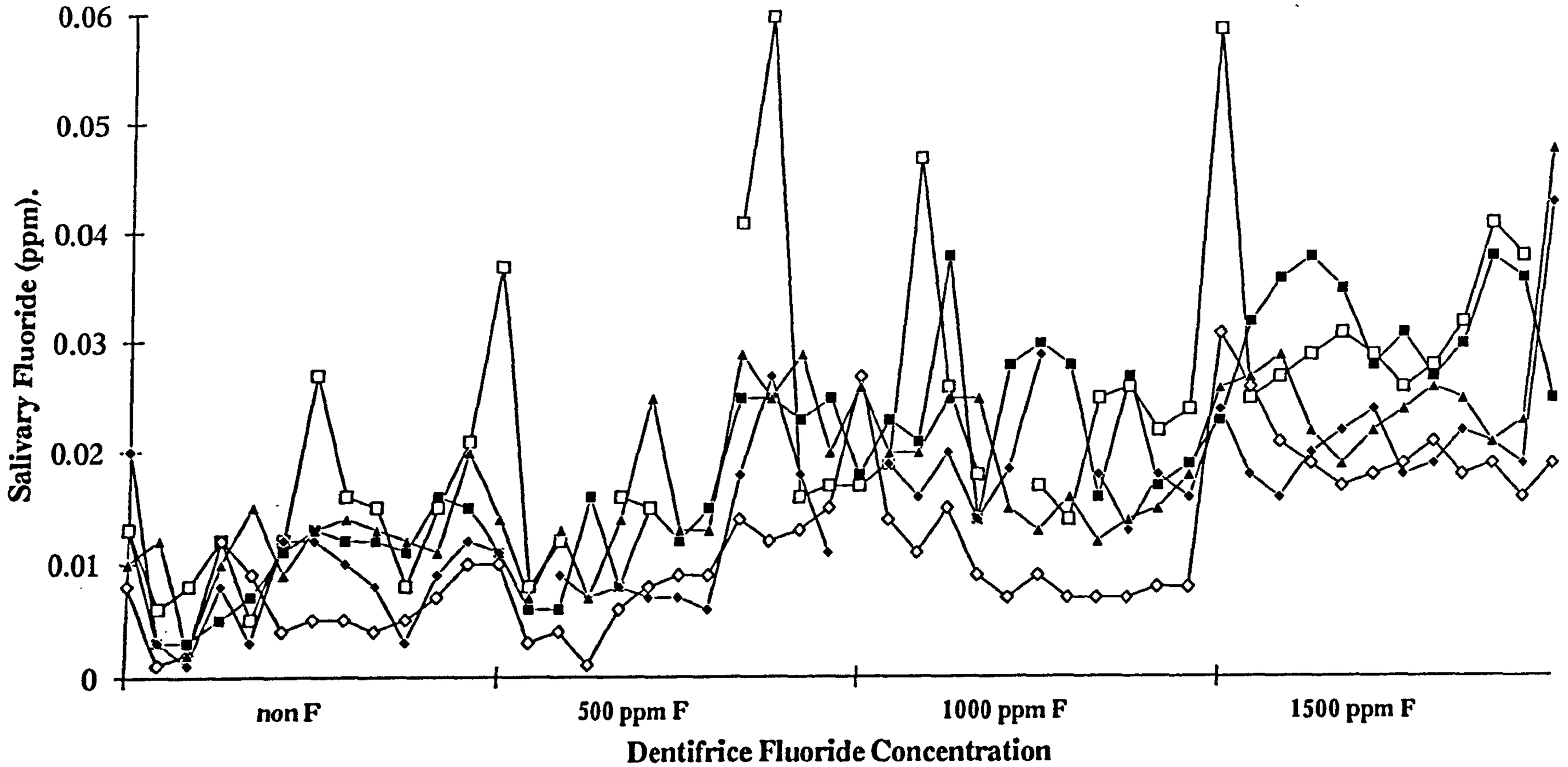
	Volunteer 13	14	15	16
	Non F Dentifrice	Non F Dentifrice	Non F Dentifrice	Non F Dentifrice
0	0.018	0.012	0.009	0.019
1	0.021	0.021	0.004	0.009
2	0.032	0.010	0.024	0.007
3	0.028	0.009	0.013	0.005
4	0.026	0.011	0.012	0.017
5	0.009	0.006	0.006	0.008
6	0.023	0.016	0.018	0.023
7	0.009	0.006	0.006	0.008
8	0.015	0.013	0.011	0.012
9	0.017	0.010	0.009	0.011
10	0.0006	0.012	0.003	0.007
11	0.023	0.012	0.011	0.011
12	0.024	0.019	0.017	0.011
	500 ppm F Dentifrice	500 ppm F Dentifrice	500 ppm F Dentifrice	500 ppm F Dentifrice
1	0.022	0.019	0.010	0.015
2	0.014	0.008	0.009	0.010
3	0.017	0.012	0.011	0.022
4	0.009	0.012	0.011	0.023
5	0.018	*	0.014	0.019
6	0.011	0.009	0.007	0.012
7	0.023	0.017	0.012	0.030
8	0.008	0.012	0.009	0.016
9	0.026	0.025	0.023	0.016
10	0.026	0.027	0.021	0.022
11	*	0.022	0.014	0.021
12	*	0.023	0.019	0.021

	Volunteer 13	14	15	16
	1000 ppm F Dentifrice	1000ppm F Dentifrice	1000 ppm F Dentifrice	1000 ppm F Dentifrice
1	0.029	0.027	0.021	0.027
2	0.028	0.042	0.026	0.024
3	0.030	0.028	0.020	0.022
4	0.029	0.028	0.015	0.019
5	0.023	0.028	*	0.031
6	0.017	0.036	0.014	0.024
7	0.022	0.024	0.012	0.018
8	*	0.025	0.015	0.020
9	0.020	0.022	0.020	*
10	0.029	0.036	0.019	0.025
11	0.024	0.030	0.018	*
12	0.022	0.028	0.018	0.030
	1500 ppm F Dentifrice	1500ppm F Dentifrice	1500 ppm F Dentifrice	1500 ppm F Dentifrice
1	0.12	0.066	0.041	0.078
2	0.063	0.042	0.044	0.065
3	0.041	0.039	0.033	0.51
4	0.046	0.031	0.036	0.049
5	0.61	0.036	0.041	0.047
6	0.043	0.041	0.032	0.039
7	0.059	0.043	0.029	0.042
8	0.062	0.039	0.027	0.051
9	0.050	0.051	0.032	0.053
10	0.048	0.056	0.037	0.051
11	0.063	0.032	0.029	0.056
12	0.13	0.068	0.035	0.054

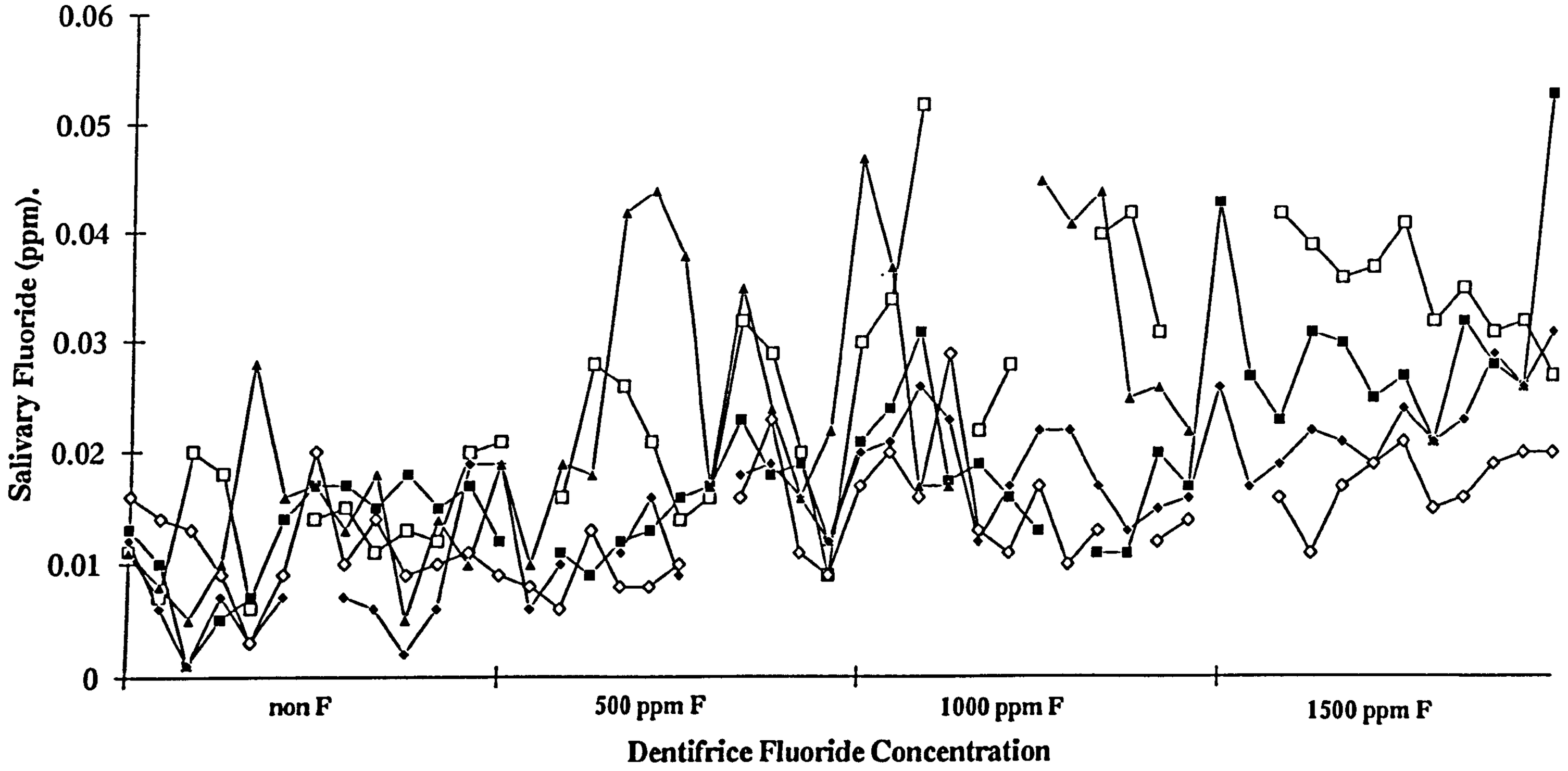
	Volunteer 17	18	19	20
	Non F Dentifrice	Non F Dentifrice	Non F Dentifrice	Non F Dentifrice
0	0.012	0.008	0.003	0.008
1	0.006	0.023	0.002	0.011
2	0.003	0.002	0.001	0.001
3	0.011	0.008	0.005	0.006
4	0.014	0.015	0.011	0.012
5	0.005	0.005	0.004	*
6	0.019	0.018	0.017	0.014
7	0.005	0.005	0.004	*
8	0.006	0.009	0.009	0.014
9	0.007	0.007	0.005	0.014
10	0.010	0.010	0.006	0.007
11	0.009	0.014	0.010	0.012
12	0.009	0.014	0.010	0.012
	500 ppm F Dentifrice	500 ppm F Dentifrice	500 ppm F Dentifrice	500 ppm F Dentifrice
1	0.016	0.006	0.013	*
2	*	0.011	0.009	*
3	0.008	0.015	*	*
4	*	0.015	0.024	0.012
5	0.017	0.010	0.00	0.033
6	0.019	0.010	0.017	0.020
7	0.014	0.017	0.045	0.020
8	0.015	0.022	0.022	0.025
9	0.015	0.022	0.022	0.025
10	0.021	0.018	0.018	0.033
11	0.020	0.018	0.018	0.039
12	0.019	0.017	0.017	*

	Volunteer 17	18	19	20
	1000 ppm F Dentifrice	1000ppm F Dentifrice	1000 ppm F Dentifrice	1000 ppm F Dentifrice
1	0.015	0.021	0.021	0.029
2	0.019	0.025	0.025	0.028
3	0.026	0.023	0.023	0.030
4	0.015	0.018	0.019	0.023
5	*	0.019	*	0.025
6	0.017	0.023	0.028	*
7	0.011	0.028	0.061	*
8	0.015	0.015	*	0.025
9	0.016	0.017	*	0.030
10	0.018	0.027	*	0.040
11	0.023	0.023	0.024	0.028
12	0.019	0.022	0.026	0.031
	1500 ppm F Dentifrice	1500ppm F Dentifrice	1500 ppm F Dentifrice	1500 ppm F Dentifrice
1	0.049	0.038	*	0.089
2	0.036	0.036	*	0.070
3	0.028	0.029	0.081	0.068
4	0.027	0.029	0.052	0.071
5	0.028	0.031	0.037	0.073
6	0.026	0.033	0.051	0.062
7	0.028	0.036	0.062	0.077
8	0.031	0.041	0.058	0.072
9	0.036	0.043	0.071	0.069
10	0.029	0.047	0.078	0.080
11	0.034	0.045	0.060	0.071
12	0.036	0.042	0.065	0.075

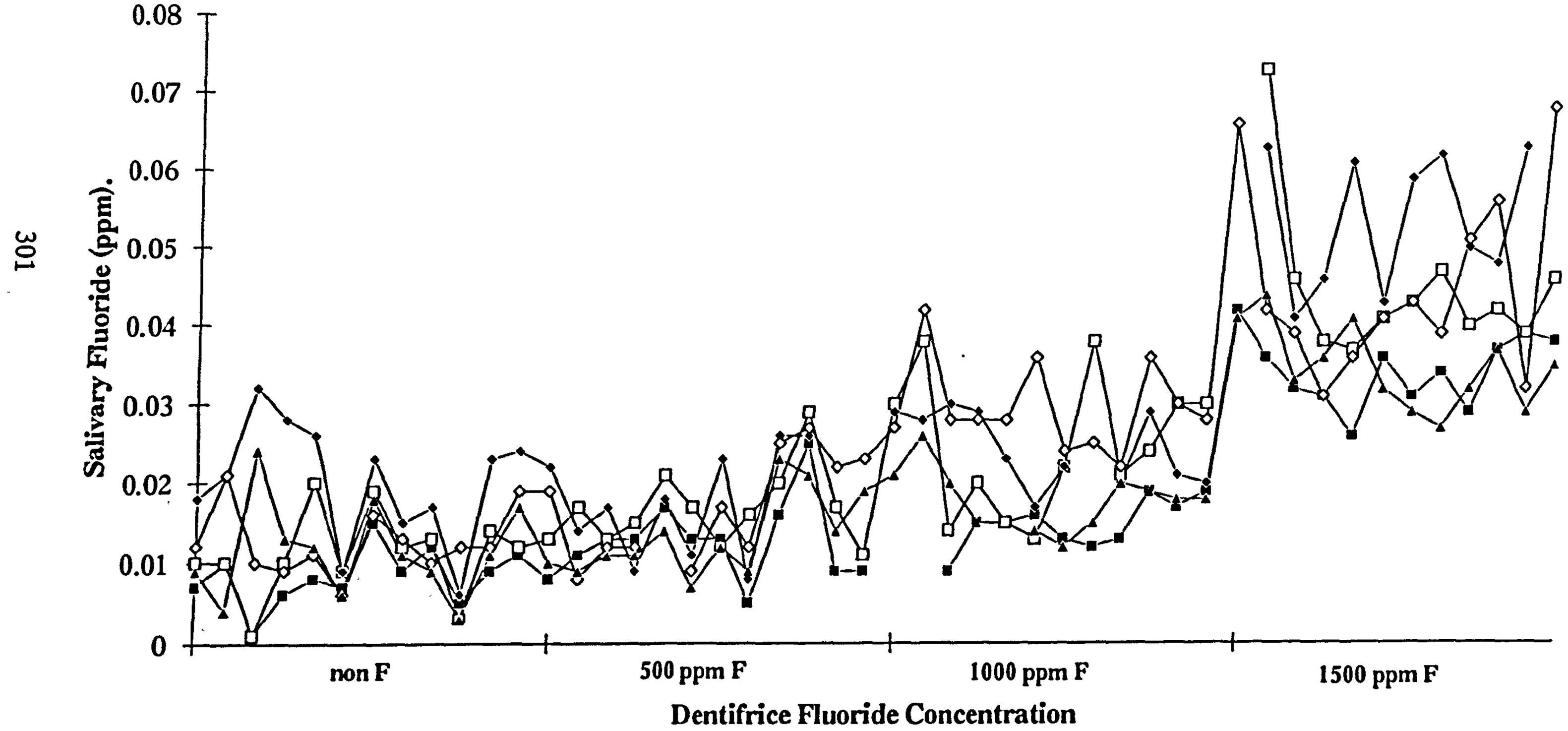
Appendix 4.2.1 Individual salivary fluoride profiles versus time & dentifrice fluoride concentration: Subjects 1, 2, 3, 4 & 5.



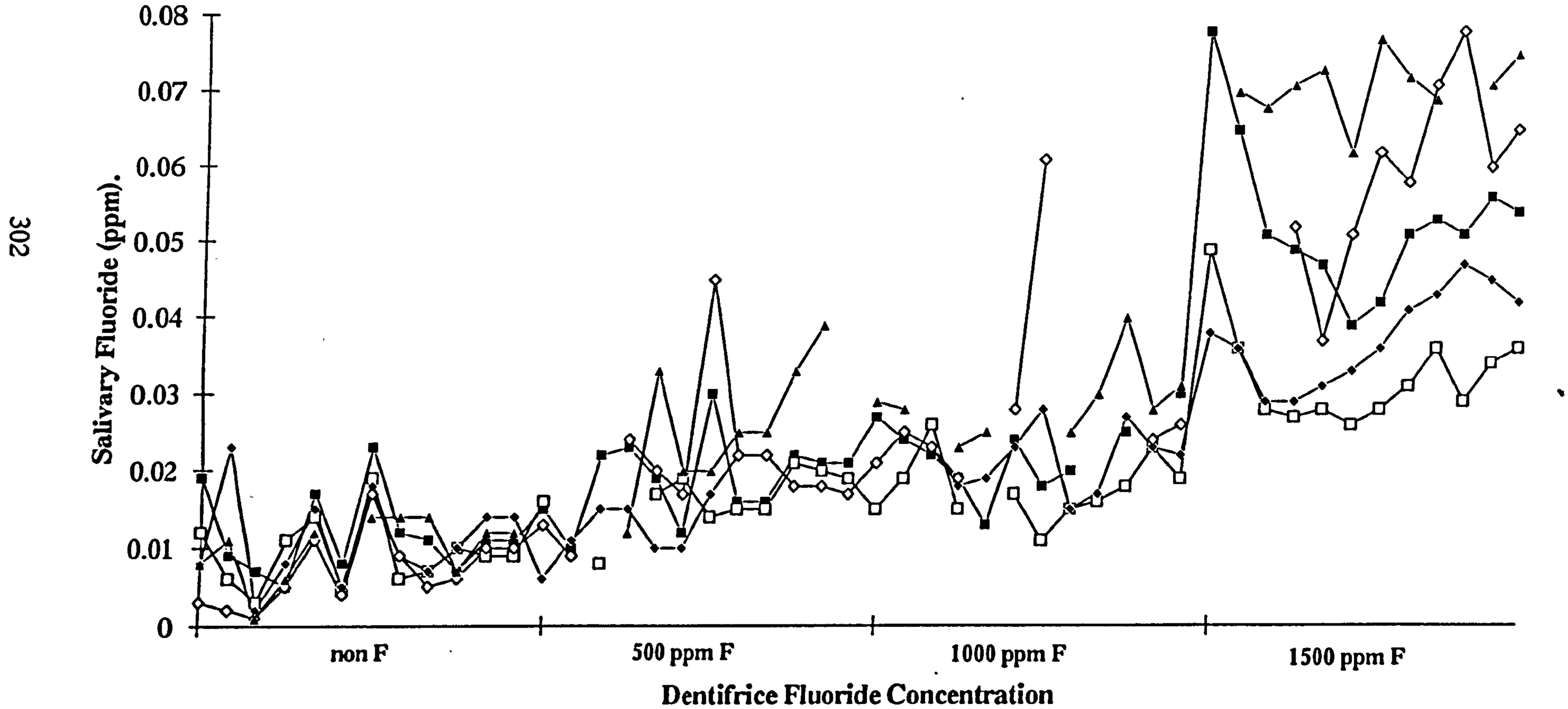
Appendix 4.2.2 Individual salivary fluoride profiles versus time & dentifrice fluoride concentration : Subjects 6, 7, 8, 9 & 10.



Appendix 4.2.3 Individual salivary fluoride profiles versus time & dentifrice fluoride concentration : Subjects 11, 12, 13, 14 & 15.



Appendix 4.2.4 Individual salivary fluoride profiles versus time & dentifrice fluoride concentration : Subjects 16, 17, 18, 19 & 20.



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