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STUDIES ON THE MICROBIOLOGY OF EARLY ENAMEL DEMINERALISATION

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DECLARATION.

This thesis is the original work of the author.

SUMMARY.

Two theories exist regarding the microbial aetiology of dental caries, namely the Specific and Non Specific Plaque Hypotheses. Almost all human studies have involved either cross-sectional or longitudinal designs, with consequent limitations associated with both techniques. Therefore, an in situ model was designed to overcome some of these problems, and while most studies have examined only the relationship between plaque microflora and enamel demineralisation, in this study, many other factors thought to influence the development of caries were also investigated. These included diet, salivary counts of Strep. mutans and lactobacilli, salivary flow rate and buffering capacity, as well as previous caries experience.

Preliminary studies examined the reproducibility of experimental methods, compared plaque microflora obtained from enamel specimens mounted on an intra-oral appliance to that from the natural dentition, and examined the susceptibility of enamel to demineralisation. Results showed little variation between repeat identifications from numerous sub-divisions of one original plaque sample. Further, no qualitative difference in microflora between plaques associated with natural and exogenously derived tooth surfaces was found. Since enamel susceptibility was found to be as variable within one tooth as between teeth, it was considered appropriate to employ sections from different teeth in subsequent studies.

As the initial work showed that factors such as subject and sequence of experimental runs affected the results to some extent, the statistical package chosen for analysis of the effect of different treatment protocols and plaque microflora on enamel demineralisation, took these other variables into account.

The in situ study was performed using seven volunteers, and attempted to determine the relationship between plaque microflora and enamel demineralisation, under normal conditions and with extra-oral sucrose stressing, both with and without inoculation of the subject's own Strep. mutans. Results showed that considerable inter-subject variation existed, in terms of plaque microflora and demineralisation. Sucrose caused no significant effect on plaque microflora composition but, overall, was

associated with a slight increase in demineralisation, compared to unstressed plaque. Success of Strep. mutans implantation was very variable, as shown by proportional counts of this organism in plaque samples, although in all subjects, isolation frequency rose following implantation. Overall, the combination of implantation and sucrose application resulted in significantly greater demineralisation.

In general, the isolation frequency of Strep. mutans was significantly higher in plaque associated with greater amounts of mineral loss, with mean and median proportions showing a similar trend. Lactobacillus spp. proportions were significantly higher in plaque associated with the greatest amount of demineralisation. Veillonella fell in mean proportion with increasing demineralisation, and no trend was seen, in this regard, in relation to Actinomyces.

However, these results relate only to microbial counts at the end of the three week experimental period. Hence, a study on the microflora of developing plaque during this period was performed. As the total microbial count increases during the early stage of plaque growth, enamel slabs, from which absolute counts can be obtained, were used, as proportional counts alone can be misleading when the total count is varying. These early studies showed a change from a Streptococcus - to an Actinomyces - dominated plaque with time, and found no difference between sucrose and unstressed plaque with regard to proportional and final absolute bacterial counts, but demonstrated that the maximum bacterial mass was achieved more rapidly in sucrose plaque.

The results of the studies in this thesis are in keeping with those of other workers, and suggest that Strep. mutans has a major role in initiation of demineralisation, while lactobacilli are associated with more extensive lesions, and may be important in the progression of established lesions. However, in some plaque samples associated with demineralisation, both Strep. mutans and lactobacilli were isolated in, at most, negligible amounts, implying that other acidogenic organisms are capable of producing demineralisation.

One of the reasons for the failure to demonstrate a direct correlation between Strep. mutans count and level of demineralisation at individual

sites may be due to variable pathogenicity of the organism, and this possibility was investigated with an in vitro study of the cariogenic potential of different strains of the organism. This showed a significant correlation between the cariogenic potential of individual strains and the natural and experimental caries experience of the subjects from whom the strains were isolated. Similarly, the DMFS scores of subjects correlated with the in situ results, but none of the other characteristics commonly used as screening tests (namely salivary microbial counts, salivary flow rate and buffering capacity, and diet) showed a significant correlation with demineralisation levels. The results suggest that in vitro assessment of cariogenic potential of Strep. mutans may be a useful additional screening test in the detection of caries-risk individuals.

The appliance model developed and tested in this thesis offers benefits over conventional experimental designs, in that rapid demineralisation can be produced by stressing test sites without affecting the subject's natural dentition. Different treatment conditions can be applied to the two sides of the appliance during each experimental run, so that the influence of general intra-oral conditions on the results will be minimised. In addition, accurate plaque sampling from discrete enamel sites is readily achievable, and the use of enamel slabs of known surface area allows microbial absolute counts, in addition to the more generally reported proportional counts, to be calculated. Finally, mineral loss can be assessed accurately by employing thin enamel sections for which pre- and post-experimental quantification of mineral content is possible.

Although the multifactorial nature of dental caries means that a definitive cause-and-effect relationship between specific microorganisms and initiation of demineralisation cannot be established in the human oral environment, the results of the studies in this thesis are in keeping with the findings of previous workers, in suggesting that Strep. mutans and lactobacilli play a greater role than other plaque organisms in the initiation and development of demineralisation. Future studies using this model, involving larger subject numbers, may provide further information regarding early plaque development and the microbial aetiology of dental caries, and in addition, the use of the appliance model may be extended, for example, to include investigation into the effect of antimicrobial agents.

ABBREVIATIONS.

A	Actinomyces
ABB	Anaerobic Blood Broth
cfu	colony forming units
Ch.	Chapter
cm	centimetre
cm^2	square centimetre
conc.	concentration
Δ	increment of
Δz	increment of total mineral loss
DMFS	decayed, missing and filled permanent tooth surfaces.
eg	for example
FAP	fluorapatite
Fig.	Figure
g	gram
h	hour
HAP	hydroxyapatite
ie	that is to say
kV	kilovolt

L.	Lactobacillus
Lact.	Lactobacillus
LB	Lesion Body
lbs	pounds
log	decimal logarithm
$\log_{10}$	decimal logarithm
m	minute
mA	milliampere
mbar	millibar
min	minute
min.	mineral
ml	millilitre
mg	milligram
mm	millimetre
mm^2	square millimetre
mM	millimole
µA	microampere
µg	microgram
µl	microlitre

μm	micrometre
MSB	mitis salivarius bacitracin agar
n	number
NCTC	National Collection of Type Cultures
nmol	nanomole
ND	not detectable
NP	normal plaque
NS	not significant
p	probability
p.	page
pH	negative decimal log of molar hydrogen ion concentration
pK_a	negative decimal log of dissociation constant
pp.	pages
ppm	parts per million
rpm	revolutions per minute
s	second
S.	Streptococcus
SD	Standard deviation
SL	selective for lactobacilli

sp.	species
SP	sucrose plaque
SPM	sucrose plaque with inoculation of Strep. mutans
spp.	species (plural)
Strep.	Streptococcus
SZ	Surface Zone
TYCSB	trypticase yeast cystine sucrose bacitracin
vol.	volume
w/v	weight per volume
wt.	weight
&	and
°C	degree Celsius
%	percent
<	less than
>	greater than
±	plus or minus
+ve	positive
-ve	negative

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction.

Dental caries is a disease of the mineralised tissues of the teeth caused by the action of microorganisms on fermentable carbohydrates (Kidd & Joyston-Bechal, 1987) resulting in localised destruction of the tooth tissues.

As the dental hard tissues are more resistant to destructive influences than any other tissue, more is probably known about its prevalence throughout the ages than with any other disease. Caries appears to have been present, although rare in Homo sapiens, since the Pleistoscene period in Europe, and during the Palaeolithic and Mesolithic periods caries was not uncommon, with one study on skulls from the Mesolithic period showing that 4 % of the teeth were carious (Hardwick, 1960). In Britain the prevalence of caries showed little variation until the Iron Age when it rose markedly but fell again in the Anglo-Saxon period following the withdrawal of the Romans and the concomitant change in diet (Moore & Corbett, 1971).

Throughout this period the most frequent site of attack was at or near the amelo-cemental junction of the tooth. By the 17th century the caries prevalence had increased again and there was evidence of a change towards the modern pattern of caries attack, with the interstitial contact areas and occlusal fissures being more frequently involved (Hardwick, 1960). These trends continued and by the second half of the 19th century the prevalence and distribution of lesions were similar to that found in the English population in the early 1980s (Holloway, 1983).

The changes were associated in time with the change in diet, with the consumption of sugar increasing from almost zero at the beginning of the 17th century to 90 lbs per head by the middle of the 19th century.

Dental caries continued to rise in industrialised countries until the 1960s, with the exception of the years associated with sugar restriction during the Second World War when a reduction in caries prevalence and severity

was found (reviewed by Sognnaes, 1948). Since 1970 there has been a reduction in the caries experience in Westernised countries (reviewed by Downer, 1984), with many studies reporting reductions of 50 % or more in the same population groups over 10-15 year intervals.

Whilst the caries rate is declining in Western countries, its prevalence and severity is increasing rapidly in underdeveloped countries (reviewed by Sheiham, 1984) and the cost of dental treatment in Britain is still one of the highest expenditures in the National Health Service (Murray, 1983).

1.2. Early Theories of Caries Aetiology.

The agent first thought to be responsible for caries lesion formation was the worm. This idea appears to have been universal at one time, and references to this have been discovered on clay tablets, from about 5,000 BC, excavated from an ancient city in the Mesopotamian area and from Chinese characters dating back to around 1,000 BC (Newbrun, 1983).

From the end of the 18th century until the middle of the 19th century, the Vital theory of tooth decay was dominant. Here it was postulated that caries originated from within the tooth itself, analagous to bone gangrene (Nikiforuk, 1985). Other theories put forward at this time included the Chemical theory of Parmly in 1819, and the Parasitic or Septic theory of Erdl (1843). The chemical theory proposed that an unidentified chemical agent was responsible for the caries, and that the process began on the surface of the enamel. Support was given to this concept by Robertson in 1835, who proposed that caries was caused by acid formed by fermentation of food particles around the teeth. The parasitic theory was based on the fact that microorganisms had been detected by van Leeuwenhoek (1632-1723) from material taken from carious cavities, and it was therefore proposed that these bacteria could cause decomposition of the tooth tissues. However no explanation was given as to how these organisms could destroy the tooth. The Chemical-parasitic theory, a blend of the above two theories, was proposed by W.D.Miller and in "The Microorganisms of the Human Mouth" (Miller, 1890) he described his theory based on his own experimental work and on previous communications from other workers. Miller identified carbohydrate as the bacterial substrate, and noted that the decalcification of enamel produced

by bacterial acids was the major factor resulting in destruction of the tissue. He failed, however, to identify plaque as the source of bacteria, and assumed that the acids were produced by the fermentation of impacted foodstuffs by salivary bacteria. G.V.Black (1898) considered that the acid attack was produced by bacteria in situ on the teeth. This was supported by Williams (1898), who observed dental plaque on the surface of enamel, and considered that this was a means of localising acids produced by bacteria in contact with the tooth, and of partially preventing the dilution and neutralisation of the acids by saliva.

1.3. Alternative Theories of Caries Aetiology.

Although current theories regarding the aetiology of enamel caries initiation are still based on the modifications by Black and Williams of the chemico-parasitic theory of Miller in 1890, a number of other proposals have been suggested. The Proteolysis theory of Gottlieb (1947) suggests that the organic element of the enamel is first attacked by proteolytic bacteria, and that the inorganic component is then subsequently lost either by acid dissolution (Frisbie & Nuckolls, 1947) or by mechanical loss of physically unbound prisms (Pincus, 1949). A number of criticisms have however been levelled against this theory, in particular the fact that the organic component comprises such a small fraction of the enamel (Jenkins, 1978).

The Proteolytic-chelation theory has been proposed by Schatz (Schatz & Martin, 1962). It is suggested that products of proteolysis of tooth substance, and possibly also of the acquired pellicle and foods, may act as chelating agents, releasing mineral ions from enamel. Whilst the amount of chelating agents released by proteolytic degradation of the small organic phase of enamel is likely to be negligible (Jenkins, 1978), calcium chelation may indeed occur; histopathological features of enamel caries can be simulated in vitro with chelating agents (Mortimer & Tranter, 1971), and many natural chelators (eg lactate and some amino acids) are present in plaque (Mørch et al., 1971).

An intrinsic concept of caries aetiology has been proposed by Jackson and co-workers (1973). They suggest that specific regions of odontoblasts within the pulp of a tooth are damaged by an auto-immune process and

conclude that caries should be regarded as a degenerative disease. This theory is based on cross-sectional epidemiological evidence and has been criticised by Edgar (1974) and Sofaer (1982) on its statistical and genetic content.

1.4. Current Theories on Caries Aetiology.

Today it is universally accepted that caries is a multifactorial process, with the development of the lesion being due to an interaction of three primary factors, the host, the microflora and the diet. For caries to occur, favourable conditions within each of these groups must exist concomitantly for a sufficient length of time, ie a susceptible host, a cariogenic flora and a suitable substrate (Fig. 1.).

Dental caries may be classified as primary enamel caries, recurrent caries associated with existing restorations, root surface and dentine caries. Many secondary factors may influence the development of lesions in these different groups, and therefore, for the purpose of discussion in this thesis the term dental caries shall relate only to primary enamel caries, in an attempt to limit the factors associated with this multifactorial disease.

1.5. Saliva and Dental Caries.

Whole saliva is a mixture of the secretions of the three pairs of major salivary glands and of the numerous minor salivary glands. It also contains gingival crevice fluid, varying amounts of bacteria, desquamated epithelial cells, leucocytes and food debris. Variations exist in the composition of the fluid secreted from the different glands, and factors such as the flow rate, duration and type of stimulus, diet and time of day the saliva is collected will also influence its composition (Mason & Chisholm, 1975).

Saliva plays a major role in maintaining the normal physiological functions of the mouth. These include mastication and deglutition of food, speech, taste sensation and the initial digestion of carbohydrates. It is also involved in the formation of the proteinaceous membrane (the acquired pellicle) on the enamel surface of the teeth, and in the initiation of plaque formation and its subsequent maturation and metabolism.

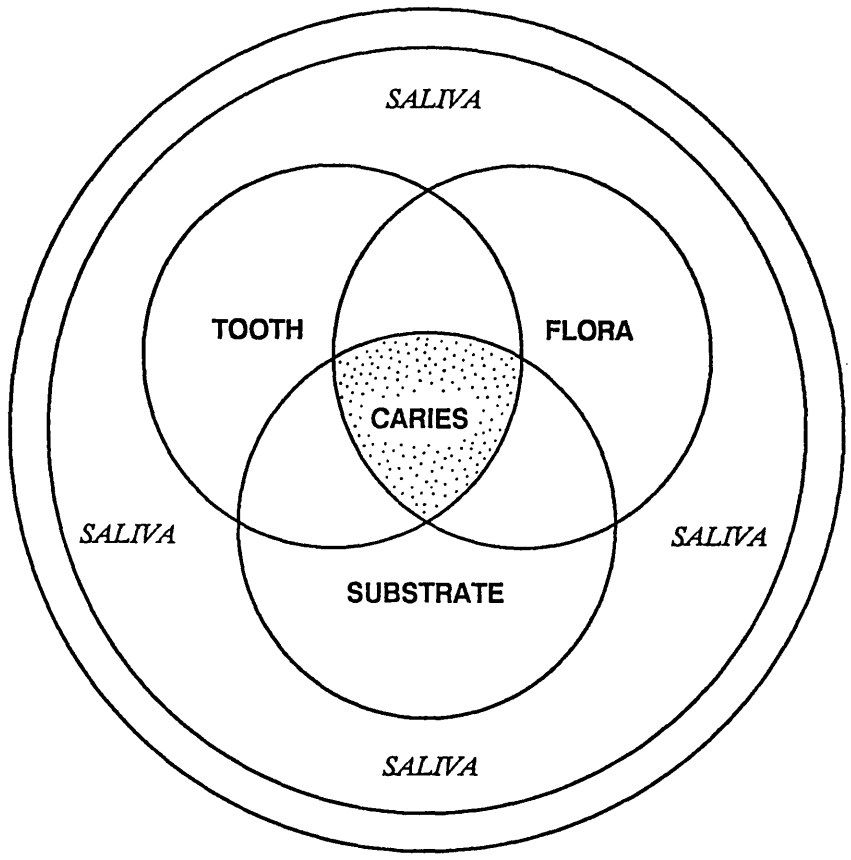


Fig. 1. **The inter-relationship of tooth, bacterial microflora and substrate which must interact over a period of time for caries to develop, with the modifying influence of saliva. Adapted from Nikiforuk (1985).**

Calcium and phosphate ions are normally present in saliva at concentrations which result in supersaturation of saliva with respect to the hydroxyapatite salt of the tooth. The presence of these ions in saliva therefore plays an important role in preventing dissolution of the enamel surface under normal resting physiological conditions.

The relationship between salivary flow rate and caries has received much attention. Studies have shown that severe impairment of salivary secretion can result in a marked increase in the incidence of caries. The extirpation of salivary glands in rats has been shown to result in rampant caries even when the animals were fed a low cariogenic diet (Weisberger et al., 1940), and in man the relationship between salivary flow rate and caries has been studied in groups of patients receiving radiotherapy in the region of the salivary glands and those with decreased flow rates due to pathological conditions such as Sjögrens Syndrome. The severe impairment of secretion is associated with an increase in enamel and root surface caries (Karmiol & Walsh, 1975; Dreizen et al., 1977; Talal, 1987). Llory and co-workers (1972) have shown that the decrease in salivary flow rate found in these patients was also associated with a change in both salivary and plaque flora with an increase in Streptococcus mutans and lactobacilli.

One of the most important functions of saliva in respect of caries prevention is the clearance of carbohydrates from the mouth. A theoretical study of salivary sugar clearance (Dawes, 1983) identified the three most important factors as being the unstimulated salivary flow rate, and the volume of saliva in the mouth both before and after swallowing. Dawes (1987) has reviewed the methods available to assess both stimulated and unstimulated salivary flow rates and the physiological factors which have been shown to influence the unstimulated flow rates in human subjects. The reduced salivary flow rate seen in patients with dry mouth reduces the sugar clearance and, presumably, this contributes to the increased caries incidence in such subjects.

Another important function of saliva is its ability to buffer both the acids produced by plaque bacteria and those entering the oral cavity directly. Of the bicarbonate, phosphate and protein buffering systems in saliva, bicarbonate is the most important, providing approximately 85 % of the

total buffer capacity (Wah Leung, 1951). The pH of saliva is governed by the ratio between the combined and free carbonic acid as expressed by the Henderson -Hasselbach equation

$$pH = pK_a + \log \frac{[HCO_3^-]}{[H_2CO_3]}$$

where $[H_2CO_3] = 0.03 \times pCO_2$ and the $pK_a \approx 6.1$.

Since the partial pressure of CO_2 in the saliva is relatively constant and in equilibrium with that in venous blood within the glands, the salivary pH is chiefly determined by variations in bicarbonate concentration (Nikiforuk, 1985). The bicarbonate concentration is low in unstimulated saliva (Dawes, 1975) but increases with flow rate, and diffusion of bicarbonate into plaque may therefore be an important mechanism by which stimulated saliva helps to redress the fall in plaque pH which occurs after carbohydrate consumption (Frostell, 1974; Abelson & Mandel, 1981). An additional buffering effect of bicarbonate is due to the change of phase of CO_2 in saliva from dissolved state to gas phase (Ericson & Mäkinen, 1986). The removal of protons by bicarbonate leads to an escape of CO_2 from saliva which in turn leads to proton loss from water and an increase in hydroxyl ion concentration and increased pH. This phase change buffering therefore confers additional buffering power to the bicarbonate system.

The phosphate buffer system functions in a similar way to the bicarbonate system, but without the additional effects due to phase change. However the concentration of the system is smaller than the biocarbonate system, and therefore its buffering power is less than that of the latter.

In addition to these buffer systems, organic substances are present in saliva which may help to reduce the effects of carbohydrate metabolism by plaque bacteria. Urea is the nitrogenous end product of protein metabolism and its concentration in saliva correlates with its blood concentration (Nikiforuk, 1985). In saliva, urea is rapidly metabolised by plaque bacteria to form ammonia and carbon dioxide, with a net production of base. This source may be an important factor in the pH-rise phase in the Stephan curve. Studies of renal patients with increased urea and ammonia salivary levels have found a decrease in caries activity even

although their fermentable carbohydrate intake was high (de Stoppelaar, 1982).

Saliva contains a number of antibacterial factors but whether or not they play a role in influencing the caries process is not clear (reviewed by Nikiforuk, 1985; Ericson & Mäkinen, 1986). The enzyme systems present include lyzozyme which can destabilise the cell membranes of micro-organisms (Mandel, 1987), resulting in their lysis, and lactoperoxidase which oxidises thiocyanate to hypothiocyanite in the presence of hydrogen peroxide. This anti-bacterial system is known to be inhibitory towards some Lactobacillus, Streptococcus and Actinomyces species (Tenovuo & Pruitt, 1984). Lactoferrin exerts a bacteriocidal effect by strongly binding to iron and therefore making it unavailable as a bacterial nutritional source. In vitro studies have shown that lactoferrin is antagonistic to Strep. mutans, but the significance of this is not known (Nikiforuk, 1985). The predominant class of immunoglobin in saliva is secretory immunoglobulin A (S-IgA). These antibodies interfere with the adhesion of microorganisms to oral surfaces (Kleinberg et al., 1979) and are the principal source of immune activity in the occlusal and bucco-lingual regions, above the cervical area of a tooth. In the gingival and approximal regions, the gingival crevicular fluid is the main source of immune activity, and here serum IgG, IgM and IgA are also present. Polymorphonuclear leucocytes, macrophages, T - and B - lymphocytes and components of the complement system are also found, and it is believed that in this region the immune system functions by interfering with bacterial adhesion, and assisting the opsonisation, lysis and phagocytosis of microorganisms.

Over the past twenty years, attempts have been made to produce a vaccine which would confer protection against dental caries (Russell & Johnson, 1987). As Strep. mutans is generally considered to be the bacterium most implicated in the initiation and development of carious lesions, attention has been focussed on developing an anti-Strep. mutans vaccine. Various components of the bacterial structure, such as glucosyl transferases and cell wall associated proteins, have been used in trials in rodents and primates, and some protection against dental caries has been found (Lehner et al., 1980). The immune process which is of importance in rats is secretory IgA, produced by salivary glands, while in primates, protection is

afforded by IgG, which is generally in the form of circulating antibodies released into the mouth via the gingival crevice, or, can be produced locally in the gingivae (Russell & Johnson 1987). Primates are considered to be a better model for humans than rodents, but there is still doubt concerning the relevance of animal results extrapolated to humans (Sims, 1985).

There is concern, also, that some cell wall derived vaccine may induce antibodies which will cross react with heart muscle, although this has not been conclusively proven (Hughes et al., 1980). Even if a vaccine of proven safety record were developed, it is unlikely that the disease would be eradicated, due to antigenic drift occurring within the Strep. mutans population (Beem et al., 1985), and the presence of other acidogenic organisms which may also be implicated in the aetiology of caries (Krasse & McBride 1984).

Furthermore, the ethical problems surrounding the use of a vaccine to control a disease which is not directly life-threatening, and the fact that dental caries should, in future, be controllable by other methods, means that vaccine research in humans has not been undertaken, as permission for such trials has not been granted (Marsh, 1988).

1.6. The Acquired Salivary Pellicle.

A thoroughly clean tooth will within seconds of exposure to saliva become covered with a thin acellular organic film called the acquired pellicle. The thickness of this film increases rapidly during the first two hours of its formation and thereafter the process proceeds at a slower rate (Sönju & Rolla, 1973). It is derived mainly from salivary proteins and glycoproteins which selectively adsorb on to the hydroxyapatite surface of the tooth. Studies have shown a similarity in the amino acid composition of pellicles removed from the enamel surfaces in various regions of the mouth, suggesting that their formation is a selective process involving only a few proteins with a high affinity for the hydroxyapatite surface (reviewed by Sönju, 1986). A chemical analysis of 2 hour pellicle by Sönju and Rolla (1973) found relatively high levels of acidic amino acids and small amounts of sulphur-containing and basic amino acids. Eggan (1982) has reported that a phospho-protein component constitutes the

major proportion of pellicle, but immunospecific investigations have indicated that proteins such as amylase, lyzozyme, albumin, glucosyltransferase and immunoglobulins may also be present in small amounts (Orstavik & Kraus, 1973; 1974; Rolla, et al., 1983). Carbohydrate analyses have shown that glucose comprises a high proportion of the carbohydrate in the pellicle. As glucose is present in only very small amounts in salivary glycoproteins, it has been suggested that the glucose in pellicle may be derived from remnants of bacterial extracellular polysaccharides (Sönju et al., 1974).

A number of functions have been attributed to the acquired pellicle. These include [i] protection of the underlying enamel surface; [ii] influencing the process of bacterial adhesion to the tooth surface; [iii] acting as a substrate for bacterial metabolism (Armstrong & Hayward, 1968), and [iv] forming a reservoir for protective ions, including fluoride.

Mayhall (1970) has shown that at least some of the pellicle is resistant to acid, and it has therefore been suggested that this proteinaceous layer may help to protect the enamel surface from bacterial and dietary attack. It has also been suggested that the proteins may act as a diffusion barrier regulating dissolution and remineralisation processes and may play a role in subsurface lesion formation (Moreno & Zahradnik, 1974).

The mechanisms involved in adsorption of oral bacteria to the acquired pellicle have been extensively studied (reviewed by Gibbons, 1980; 1984) and are believed to involve specific lectin-type interactions between structures on the surface of the microorganisms and the salivary coat. Rolla and co-workers (1983), have suggested that in addition, the minor protein components present in pellicle such as immunoglobulins, lyzozyme, albumin and glucosyltransferase, will also influence the binding of bacteria to the saliva-coated hydroxyapatite surface. The microorganisms involved in the early colonisation of the cleaned tooth surface and the proposed mechanisms involved in their selective adsorption to the pellicle layer will be discussed in the following section.

1.7. Dental Plaque Formation and Composition.

1.7.1. Bacterial Adsorption to the Pellicle Coated Surface.

Dental plaque can be defined as a dense, non-calcified bacterial mass, so firmly adherent to the tooth surface that it resists removal by salivary flow or rinsing.

Immediately following the cleaning of a tooth surface in the oral cavity, salivary proteins and glycoproteins are selectively adsorbed on to the enamel surface to form the acquired pellicle as described in Section 1.6. After only a short period of exposure to the normal oral environment, bacteria become adsorbed to this proteinaceous layer. This adsorption is believed to be a selective process with some bacterial species being adsorbed from saliva to a much greater extent than others, even although the former group are present in much lower proportions in saliva (van Houte et al., 1970; 1971; Liljemark & Gibbons, 1971; 1972). This is in contrast to a previous theory which suggested that a non-selective "entrapment" of bacteria was involved in the initial colonisation of the tooth (reviewed by Gibbons & van Houte, 1973).

The first phase in the adsorption of an organism is thought to be a loose association between the bacterium and the pellicle-covered tooth surface due to attractive van der Waal's forces (Gibbons, 1984). However as the bacterium approaches the surface it is repelled by the negative electrostatic charges possessed by most natural surfaces and by most bacteria. This results in a state of equilibrium, with a balance between the attractive and repulsive forces, and with the organism held at a distance of approximately 10 nm from the surface (Gibbons, 1984). Adsorption at this stage is reversible, but if this space is bridged by macromolecules which can link both together by hydrogen, electrostatic or hydrophobic bonds the attachment then becomes firmer (Gibbons et al., 1985). This type of adhesion has been classified as non-specific. In contrast, if stereochemical interactions take place which involve complementary components of the bacterium and of the pellicle surface to form a "lock and key" mechanism, then specific adhesion is considered to have occurred. Observations suggest that bacteria possess on their surface, lectin-like ligands, called adhesins, which can interact in a stereochemical manner

with receptors on the oral tissues (Gibbons, 1984). Both non-specific physical bonding and specific stereochemical interactions are believed to be involved in the attachment of bacteria to the acquired pellicle covering the tooth surface (Gibbons, 1980; Nesbitt, 1982).

Several studies have suggested that the hydrophobic properties of bacteria play an important role in their attachment to the teeth (Nesbitt et al., 1982; Gibbons & Etherden, 1983; Rosenberg et al., 1983). Gibbons and co-workers (1985) surmised that pellicles contain multiple binding sites for specific strains of organisms, some of which are high-affinity and some low-affinity sites. They suggested that, although bacteria may attach to pellicles without the involvement of stereochemical adhesin-receptor interactions, the strength of these bonds is low and probably inadequate to permit colonisation. However simultaneous involvement of stereochemical interactions greatly increases the strength of the bond. They also considered that the high degree of selectivity involved in bacterial attachment to the pellicle surface could not be explained solely by physical forces which promote only non-specific adhesion, but must also involve specific stereochemical interactions.

The bacterial adhesins involved in these specific interactions are frequently present in filamentous surface appendages such as pili or fimbriae (Gibbons, 1984). The composition of these lectin-like adhesions present on individual bacteria has been extensively studied (Ellen et al., 1980; Cisar et al., 1981; Gibbons & Etherden, 1982) and the differences in their components is thought to be responsible for the high degree of specificity involved in the initial colonisation of the pellicle-covered tooth surface.

1.7.2. Early Plaque Colonisation.

The first stage in the bacterial colonisation of surfaces in the oral cavity requires the organism to adsorb to the surface to prevent washing away by the flow of secretions. Studies of the adherence and subsequent colonisation of the acquired pellicle by oral bacteria in vivo have found that certain species predominate in the early stages of plaque formation (Carlsson, 1967; Ritz, 1967; Socransky et al., 1977; Moore et al., 1982). The adherence and early colonisation of oral streptococci has been

extensively studied, and it has been found that certain streptococcal species adhere with high affinity and in high numbers to in vitro salivary pellicles, and that these findings correlate with their in vivo adherence and subsequent colonisation of cleaned tooth surfaces (van Houte et al., 1971; Gibbons & van Houte, 1980). The streptococci which initially adhere to and colonise the pellicle layer in high numbers belong to the Strep. sanguis and Strep. mitis species. Actinomyces species are also normally present, in much lower proportions, amongst the earliest microbial colonisers, and during the first 24 hours of plaque formation up to 90 % of the organisms belong to either streptococcus or actinomyces (Nyvad & Fejerskov, 1986). Other species have, however, also been found in relatively high proportions in plaque less than 48 hours old. These include Veillonella spp., Neisseria spp. and Haemophilus parainfluenzae (Ritz, 1967; Kilian et al., 1976; Socransky et al., 1977).

Liljemark and co-workers (1986) investigated the relative proportions of bacteria in saliva and 2 hour plaque. They found that Strep. sanguis and Actinomyces viscosus were present in much higher proportion in plaque than saliva suggesting that salivary pellicle strongly promoted the adherence of these species.

Socransky and co-workers (1977) have divided the development of plaque on cleaned tooth surfaces in vivo into three phases. The first, termed the phase of initial colonisation, spans the period from 0 to 8 hours, and during this interval total counts of 10^5 to 10^6 organisms per cm^2 enamel have been found (van Houte et al., 1971). Scanning electron microscopy of these surfaces has shown that, after four hours' exposure to the oral environment, only a few coccoid or cocco-bacillary organisms adhere to the enamel surface, residing in pits or other depressions in the surface (Saxton, 1973; Nyvad & Fejerskov, 1987). By eight hours, localised microcolonies of coccoid and rod-shaped bacteria are present, sheltered by the perikymata, whilst the rest of the surface is only very sparsely colonised by organisms.

During this phase bacteria are adsorbed on to the pellicle-coated enamel surface from saliva. Studies with Strep. sanguis and Strep. salivarius (van Houte, 1976) showed that during the first few minutes, the proportions of species present on the tooth surface are similar to those found in saliva.

However, a proportional shift then takes place, with those species which have a high affinity for the surface increasing, whilst those with low affinity appear to be desorbed from the surface. This suggests that initially the bacterial adsorption to the surface may involve a reversible phase of weak association before a firmer attachment occurs.

The presence of bacterially-derived extra-cellular polysaccharides in the plaque matrix may influence the attachment of certain species, and early studies suggested that glucan, synthesised from sucrose, was required for the attachment of the mutans group of streptococci to the teeth (reviewed by Hamada & Slade, 1980). However, Gibbons and co-workers (1986) found that glucan synthesis enhanced the attachment of only some of the species belonging to this group, and suggested that different adhesins present on bacteria, and different pellicle receptors are involved in the attachment of these species to the surface.

During the second phase, rapid growth of the plaque occurs. Bacteria proliferate from the edges of the microcolonies to form monolayers which eventually fuse with each other. After one day, the surface of the microbiota is mainly composed of coccoid bacteria, with only a few filaments present. By 48 hours, the surface is completely covered by deposits of coccoid and filamentous bacteria, with the latter lying perpendicularly to the tooth surface in between islands of coccoid microcolonies (Nyvad and Fejerskov, 1986). During this phase total counts of 10^8 organisms per cm^2 are reached (Socransky et al., 1977).

The rapid increase in bacterial numbers seen during this period is thought to be due mainly to growth of the organisms, with changes in the proportions of species taking place due to differences in the rates of cell division, release and death (Beckers & van der Hoeven, 1982). Between eight hours and one day an increase in coccoid organisms and a fall in the proportion of filamentous bacteria is seen. Socransky and co-workers (1977) have shown an increase in Strep. sanguis levels and a decrease in the proportion of Actinomyces viscosus during this period. They suggest that this is because Strep. sanguis becomes firmly attached earlier and initiates cell multiplication sooner than Actinomyces viscosus.

Coaggregation is also believed to play a role in early plaque formation.

Studies have shown that many oral bacterial species are able to adhere to each other (Liljemark et al., 1986), and it is thought that some organisms may become incorporated by attaching to other bacteria already present in the plaque. The importance of this mechanism in the early colonisation of plaque is uncertain, however, and it is felt that in plaques older than 8 - 12 hours, the main factor governing the increase in the cell number of species is likely to be cell growth rather than coaggregation (Socransky et al., 1977; Orstavik, 1984).

The third phase of plaque development begins after 48 hours. As the plaque ages, changes in the composition of the flora take place with a shift from a streptococcus- to an actinomyces- dominated plaque (Syed & Loesche, 1978).

The microbial succession seen is believed to be due to alterations in the environment within the plaque, with changes in the availability of nutrients and in the oxidation - reduction potential of the plaque taking place. These characteristics may affect bacterial growth and metabolism at different thicknesses of plaque (Marsh & Keevil, 1986a) and account for the shift from a plaque dominated by aerobic and facultatively anaerobic species, to one where facultatively anaerobic and obligatory anaerobic organisms predominate after a period of nine days (Ritz, 1967).

Considerable variation exists in the composition of mature plaque between different sites in the oral cavity. This is due in part to differences in the physical characteristics and orientation of the tooth surfaces. In addition to variation existing in the composition of the flora between fissure and smooth surface plaques, considerable differences can also occur in the microbiota isolated from adjacent sites on the same surface (reviewed by Johnson & Murphy, 1983). The composition of the flora isolated from mature plaque on approximal and fissure sites will be discussed in a later chapter.

1.7.3. Composition of Plaque Matrix.

Although the chemical and microbiological composition of plaque varies in different sites within the oral cavity, certain aspects of its structure are

relatively constant. Whole plaque consists by volume of approximately 70 % cellular material and 30 % matrix, with an overall water content by weight of 82 %, of which 50 % is intracellular and 32 % extracellular (Jenkins, 1978).

The main constituents of the plaque matrix are proteins, glycoproteins derived from saliva, and bacterial polysaccharides. A number of theories have been put forward regarding the origin of these proteins, and these include:

i] Mucin precipitation due to the acidification of saliva, or the local production of acid by bacterial deposits on the teeth.

ii] The removal of sialic acid residues from the carbohydrate side chains of salivary glycoproteins by bacterial neuraminidase (Leach, 1970).

iii] Localised increases in calcium concentration (either from crevicular fluid or existing plaque) resulting in precipitation of proteins.

iv] Aggregation of the residual protein cores of glycoproteins depleted of their carbohydrate side-chains by bacterial enzymic activity (Leach, 1980).

Certain oral bacteria are able to produce extracellular polysaccharides from carbohydrate; Marsh and Keevil (1986b) have listed these microbial populations, together with the polymers they produce. The formation of extracellular polysaccharides from sucrose by Strep. mutans has been extensively studied (reviewed by Hamada & Slade, 1980) with the products falling into two main groups, glucans and fructans, which are polymers of glucose and fructose respectively. Extracellular enzymes called glucosyltransferases and fructosyltransferases are involved in the formation of these polymers, and cleavage of the high-energy disaccharide bond of sucrose allows the polymerisation to occur without any further input of energy being required. Two types of glucans are formed by Strep. mutans - one type called "dextran" has α(1-6) core linkages with branches of α(1-2), α(1-3) and α(1-4) linkages, whilst the other called "mutan" has a core α(1-3) linkage with branches at α(1-4) and α(1-6) (Nikiforuk, 1985).
Many of the extracellular polysaccharides formed from sucrose play an important role as structural polymers of the plaque matrix. Carlsson &

Egelberg (1965) showed that plaque formed on sucrose rich diets were heavier and thicker than those formed during glucose supplementation. This was presumably due to an increase in the production of extracellular polysaccharides which may promote adhesion between bacteria (Gibbons, 1980) and increase the amount of extracellular matrix in plaque. It has been suggested that glucan acts as a diffusion barrier in plaque and may thus influence the carious process by affecting the rate of acid diffusion outward, or the movement of sugars or salivary buffers into the plaque (Critchley et al., 1967).

Whilst some models suggest that plaque thickness influences the pH found at its inner surface following sucrose challenge (Dawes & Dibden, 1986), and that the presence of extracellular polysaccharides enhances the enamel demineralising potential of plaque (Zero et al., 1986), other workers have shown no correlation between the insoluble polysaccharide content of plaque and its diffusion properties (Dibden et al., 1983), and studies on diffusion coefficients in plaque suggest that the rate of acid diffusion is unlikely to be responsible for the prolonged lowering of plaque pH following exposure to dietary carbohydrate (McNee et al., 1982).

The extracellular polysaccharides may act as an energy source for plaque bacteria when no other substrate is available. Fructans are generally highly soluble and can be readily degraded by the organisms. In addition, polymers of mutan- and dextran- type may also be broken down and used by the plaque bacteria (Carlsson, 1986).

Another important function of the extracellular polysaccharides involves their role in bacterial adhesion and aggregation. Numerous in vivo and in vitro studies have shown that the presence of sucrose markedly facilitates the colonisation of Strep. mutans on the teeth or other solid surfaces (reviewed by Hamada & Slade, 1980). The mechanism is believed to involve the complex interaction of glucan, glucosyltransferase and the surface pellicle. However a recent study by Gibbons and co-workers (1986), investigating the attachment of Strep. mutans and Strep. sobrinus species to pellicle, found that the presence of glucan and glucosyltransferase enhances the attachment of only the Strep. sobrinus strains. They therefore concluded that the mechanism was highly selective, and

suggested that the strains of Strep. mutans under investigation lacked a functional glucan binding adhesin.

In addition to cell-to-surface adherence, extracellular polysaccharides are also thought to be involved in cell-to-cell binding, and to play a role in the formation of dental plaque, as described in 1.7.2. Many strains of Strep. mutans agglutinate in the presence of dextrans, and these high molecular weight polymers are also believed to enhance aggregation between different types of bacterial cells; Bourgeau and McBride (1976) have shown aggregation between Actinomyces viscosus and sucrose-grown Strep. mutans and Strep. sanguis cells, and to glucans elaborated by these streptococci.

Jenkins (1978) stated that the most relevant area to study regarding the effect of plaque on the tooth would be the composition of the aqueous phase of the innermost layers of plaque, in contact with the enamel or its pellicle. Although this is not possible, the fluid phase of the plaque as a whole has been investigated and some of its constituents estimated (Edgar & Tatevossian, 1971; Tatevossian & Gould, 1976). The osmotic pressure of the fluid is higher than that found in plasma or saliva, and the concentration of most ions, including calcium and phosphate is much greater than those in the gingival fluid or saliva. The environment of the plaque therefore appears to be quite distinct from that expected on the basis of free equilibration with saliva.

The problems involved in attempting to extract the fluid phase from the plaque as a whole, and the fact that micro-environmental differences in the distribution of solutes within the plaque layers cannot be measured (Tatevossian & Gould, 1976), means that only limited information is obtained from studies on the aqueous phase of plaque, and that most of the data available on the effects that the surrounding oral fluids have on the physico-chemical integrity of enamel are based on salivary estimations (Larsen & Bruun, 1986).

1.8. Plaque Metabolism.

The main source of nutrients to the oral microbiota is mixed saliva (Carlsson, 1984). Under resting conditions, the concentration of carbo-

hydrates in saliva is low, with nitrogenous bases comprising a significant proportion of the available nutrients (Sreebny, 1984). However, sugar levels may increase 1000-fold following the intake of food, and in order to gain maximum benefit from these conditions and to prevent the sudden changes in sugar concentration from killing the bacteria, careful regulation of carbohydrate uptake and metabolism is required. Studies have shown that oral streptococci adapt to their "feast and famine" existence on the teeth by regulating their sugar metabolism at three levels; i] the transport of sugar into the bacterium, ii] the glycolytic pathway and iii] the conversion of pyruvate into metabolic end-products (Carlsson, 1984).

Specific protein carriers present in the cell membrane are required for the transport of the various carbohydrates into the cell, with usually more than one carrier-system existing for each sugar. In the case of glucose, streptococci have two such systems, a phosphoenolpyruvate : sugar phosphotransferase system, and a protein-linked active sugar transport mechanism. The former system has a high affinity for the sugar and dominates when the sugar concentration is low, whilst the latter system operates at higher concentration and lower pH (Carlsson, 1984). There are at least two transport systems for sucrose in Strep. mutans. At low concentration, it may enter the cell as sucrose-6-phosphate via its own phosphotransferase system (Slee & Tanzer, 1979), and subsequently be broken down into fructose and glucose-6-phosphate by sucrose-6-phosphate hydrolase, a constitutive enzyme, with the glucose-6-phosphate then entering the Embden-Meyerhof pathway. At higher sucrose concentration, a protein-linked system is believed to operate (Ellwood & Hamilton, 1982). In addition, the sucrose may be acted upon by extracellular enzymes to produce glucan and fructan polymers as described in Section 1.7.3., or may be hydrolysed by plaque fluid invertase activity (Tatevossian, 1982) with subsequent bacterial uptake of the constituent sugars.

The metabolism of carbohydrate inside the bacterial cell is the most important reaction regarding the aetiology of caries, since the metabolic end-products formed are responsible for creating the acidic environment which may result in the dissolution of tooth enamel. In most bacteria, glucose is degraded by glycolytic enzymes via the Embden-Meyerhof pathway, with the production of two pyruvate molecules from each molecule of

glucose. These reactions are carefully controlled, and are influenced by the availability of the sugar, with pyruvate kinase being the main regulatory step in streptococci (Iwama & Yamada, 1980). The pyruvate formed by glycolysis can be degraded further in a number of ways depending on whether the bacterial strain is homo- or heterofermentative, and on the concentration of substrate which may affect enzyme activity. Under conditions of sugar starvation, pyruvate is converted into ethanol and acetic and formic acids by the pyruvate formate-lyase pathway. However if the substrate is present in excess, many bacteria including strains of streptococci, lactobacilli and bifidobacteria are able to prevent the accumulation of toxic metabolic intermediates in the cell by the activation of systems which result in an increase in the glycolytic rate and more rapid drainage of products from the cell. This mechanism, activated by increased levels of fructose-1,6-diphosphate, switches on the lactate dehydrogenase pathway and results in the conversion of pyruvate into lactate molecules (Carlsson, 1986).

In addition to the levels of substrate, other factors may influence the composition and proportion of acids present in the plaque. Veillonella species are able to metabolise lactate into acetic and propionic acids (Mikx & van der Hoeven, 1975; Distler & Kröncke, 1980) and other bacteria, including members of Propionibacterium, Eubacterium and Arachnia species, are also able to metabolise lactate. The overall result of carbohydrate catabolism is therefore the production of a number of acids, the presence of which have been confirmed by a number of workers (Geddes, 1975; Distler & Kröncke, 1983).

The production of acid following the exposure of plaque to carbohydrate has been extensively investigated, both by measurement of pH and by qualitative and quantitative analysis of acid anions (reviewed by Geddes, 1984). Three methods exist for monitoring the pH of plaque following the fermentation of carbohydrate in situ:

i] The probing method allows direct measurement of discrete plaque samples in situ (Stephan, 1940) using either antimony or glass electrodes.

ii] The indwelling electrode technique (Graf & Mühlemann, 1966)

permits continuous pH measurements from electrode systems housed in a denture assembly.

iii] The sampling method employs an external electrode system to measure the pH of plaque removed from the teeth after in situ carbohydrate fermentation (Fosdick et al., 1941).

Numerous studies involving one or more of these methods have shown that bacterial exposure to carbohydrate results in a rapid fall in plaque pH followed by a gradual return to resting values over periods approaching sixty minutes, generally described as a "Stephan Curve".

A number of organic acids are produced following the fermentation of sugars by plaque bacteria, and may contribute to the pH response. The methods available for the identification and quantification of the acids produced have been reviewed by Geddes (1984), and have shown that at resting pH values acetic acid dominates the spectrum with lactate levels being low. Formic, propionic, succinic and butyric acids may also be present in low concentration. When plaque is exposed to carbohydrate, a significant increase in lactate concentration occurs, often accompanied by a fall in acetate.

The acid spectrum present in plaque may influence carious lesion development. As lactate is the main acid produced on exposure of plaque to sugars and is one of the strongest acids formed (pK 3.86), its production is therefore regarded as being crucial in relation to the development of caries (Carlsson, 1986). At resting plaque pH values, the acids found in plaque are almost completely dissociated. However, lactic acid production following carbohydrate metabolism results in a fall in pH, with the result that some of the high pK acids may then be present in their undissociated form. These acids, in particular acetate which is usually present in relatively high concentration, may therefore contribute to the buffer capacity of plaque and help reduce the fall in pH following exposure to carbohydrate (Vratsanos & Mandel, 1982). The direct role of the high pK acids in the carious process is still uncertain. Featherstone and co-workers (1979) have suggested that acetic acid diffuses into enamel in its unionised form at low pH and then dissociates into hydrogen and acetate ions which may attack the enamel crystals. This hypothesis has been supported by Geddes and co-workers (1984) who showed that acetic

acid was taken up by enamel, in preference to lactic acid, in an acid-only system, and a plaque incubation model, and Featherstone and Rodgers (1981) have shown that lactic and acetic acids are able to produce artificial carious lesions, with mixtures of the two acids having additive demineralisation properties.

1.9. Diet and Dental Caries.

Dietary factors may affect the teeth at two levels; first, while the tooth is still forming, and then, once it has erupted into the mouth.

The pre-eruptive influence on the enamel of levels of vitamins and minerals is still uncertain, but it is thought that their effect is likely to be small, with fluoride being the main dietary component influencing the resistance of the developing tooth (Rugg-Gunn, 1983). The post-eruptive effects of diet appear to be more important in relation to dental caries, with fermentable carbohydrate being the most important dietary factor.

Since practical and ethical difficulties are encountered when attempting to carry out human clinical studies relating diet to caries, a variety of sources have been used to obtain evidence linking dietary factors to the initiation or progression of this disease. These include i] epidemiological surveys, ii] human clinical studies, iii] animal experiments and iv] plaque pH studies.

1.9.1. Epidemiological Surveys.

Numerous epidemiological surveys have investigated the caries experience of groups of people before and after an increase in sugar consumption. Evidence is available from studies on the change in diet which occurred in the English population from Medieval to modern times, and from more recent changes in the availability of sugar in developing countries and remote island communities. Although many of the surveys have been criticised for a number of reasons, including the involvement of different non-standardised examiners, these world-wide studies have shown a close parallel between increased sugar consumption in many communities and an increase in caries prevalence and severity (reviewed by Rugg-Gunn, 1983).

Studies involving groups of people eating low amounts of sugar, eg, the Hopewood House Children in Australia (Harris, 1963), those affected by the war-time restriction of sugar and individuals suffering from diseases such as Hereditary Fructose Intolerance, which necessitates a low sugar intake, have shown a lower caries experience compared with other groups (reviewed by Newbrun, 1982). However, groups with increased exposure to sugar, such as confectionary workers (Anaise, 1978), and children on long-term sucrose-based medication (Roberts & Roberts, 1979) have been found to have a higher caries experience.

1.9.2. Human Clinical Studies.

The most famous human clinical study of caries was carried out in Sweden between 1945 and 1953 at the Vipeholm Hospital, an institute for mentally defective individuals (Gustaffson et al., 1954). The aim of the study was to investigate the relationship between diet and dental caries in a longitudinal manner, under relatively well-controlled conditions. The patients were divided into six main test groups and one control group, and a total of 436 individuals completed the study. The main purpose of the study was to investigate how caries activity was influenced by the addition of large sucrose supplements in sticky or non-sticky form, either with or between meals.

The main conclusions of the study were:

i] The consumption of sugar, even at high levels, was associated with only a small increase in caries increment when taken only at meal times.

ii] The consumption of sugar, between meals as well as at meal times, was associated with a marked increase in caries increment.

iii] The increase in caries activity was greatest when the sugar was taken between meals in a sticky form.

It is very unlikely that a comparable study will ever be repeated, since it is now considered unethical to alter diets of humans experimentally in directions likely to increase caries. Although some aspects of this study have been criticised, in particular the fact that the adult mentally

deficient patients who were involved were not matched for age or previous caries experience, and were given abnormally high levels of sugar supplements, it is felt that the main conclusions from the study are valid (Rugg-Gunn, 1983). Much of present-day dietary advice is based on the results of this study (Kidd & Joyston-Bechal, 1987) with emphasis being directed to reducing the frequency of intake of sugar, and advising against the consumption of sticky sweet foods.

Another large scale experiment on caries in humans was carried out in Turku, Finland (Scheinin & Mäkinen, 1975), the aim being to study the effect on dental caries increment of nearly total substitution of sucrose in a normal diet with either fructose or xylitol. The results of the study showed that both sucrose and fructose were cariogenic, whilst the replacement of sucrose by xylitol resulted in a substantial reduction in caries incidence. Microbiological studies showed that xylitol did not affect the proportion of major bacterial groups in dental plaque, but did reduce the number of most organisms, especially the acidogenic and aciduric flora, including Strep. mutans; no adaptation by plaque organisms to produce acid from xylitol was observed during the two-year study.

The results from this investigation suggest that it may be possible to replace sucrose with substances which will impart sweetness but are not cariogenic, and there is now increasing interest in the use of these sweetening agents in an attempt to reduce the cariogenic challenge to the teeth (Kidd & Joyston-Bechal, 1987).

Short-term studies involving human volunteers have been carried out, in which reversible optical changes in enamel have been produced following the rinsing of the mouth with sucrose solutions for periods of 14-23 days, associated with cessation of oral hygiene (von der Fehr et al., 1970; Geddes et al., 1978). These studies support the importance of frequency of exposure of the mouth to fermentable carbohydrates in the induction of caries, but problems with sample size and high inter-subject variation suggest that it would not be practicable to use this model to study the relative cariogenicity of different foods in the oral environment. These studies will be further discussed in a later chapter.

1.9.3. Animal Studies.

The importance of the local effect of diet on the mouth was conclusively demonstrated by Kite and co-workers (1950) who fed cariogenic diets to rats via either the normal oral route or by stomach tube and showed that caries developed only in the former group. Animal models have also i] confirmed the positive correlation between frequency of sugar intake and caries severity (König et al., 1968); ii] studied the effect of different sugar concentrations on the levels of caries (Huxley, 1977), and iii] compared the cariogenicity of different types of sugars. Such work has shown that sucrose, glucose, fructose, galactose, lactose and maltose are all cariogenic, with sucrose being the most cariogenic, whilst conflicting results have been obtained for starch, depending on whether or not is has been cooked prior to testing (reviewed by Rugg-Gunn, 1983).

1.9.4. Plaque pH Studies.

The measurement of plaque pH before, during and after the consumption of food by humans has been used to assess the acidogenic potential of different foods. The pH can be measured using one of the methods described in 1.8 and a "Stephan Curve" produced by plotting pH against time. Many surveys of snack foods and plaque pH have been carried out (Edgar et al., 1975; Rugg-Gunn et al., 1978), and in Switzerland, confectionary which causes the pH to fall no lower than 5.7 when assessed by the indwelling electrode technique, may be classified as "safe for teeth" (Mühlemann, 1969; Imfeld, 1977).

The relative acidogenicity of different sugars and different concentrations of the same sugar can also be assessed using this method, and results show that a range of acidogenic potentials exist, with some sugars having similar acidogenicity to sucrose (Imfeld, 1977).

It has recently been suggested that when testing the cariogenic potential of food-stuffs, by means of the plaque pH method, comparison should be made with positive and negative controls, and the use of sucrose and sorbitol, respectively, have been recommended for this purpose by the Scientific Consensus Conference on Methods for Assessing the Cariogenic Potential of Food (1986).

Plaque pH measurements determine only the acidogenic potential of the tested foods, and although this may be expected to correlate with the cariogenic potential, other factors may influence whether or not demineralisation will occur (Rugg-Gunn, 1983). Foods may also be tested using in vitro models, which involve the incubation of the test item with a slurry of plaque or cariogenic bacteria, such as Strep. mutans, in the presence of an enamel slab (Primrose et al., 1988). Although this method does not represent the natural oral situation, it allows the demineralising potential to be assessed as well as the acidogenic potential, and may be useful as an initial screening test, to be used in conjunction with other models.

1.9.5. The Importance of Sucrose and Other Dietary Factors in Caries Aetiology.

Although many dietary carbohydrates can be metabolised by plaque bacteria to produce organic acids, the question as to whether some sugars, in particular sucrose, are more cariogenic than others, has received much attention. Evidence supporting the increased cariogenic potential of sucrose is derived from the fact that its presence in the diet leads to increased extracellular polymer production, thus favouring plaque accumulation, and that most animal studies have shown it to be more cariogenic than monosaccharides or other disaccharides. However, plaque pH studies have found that some carbohydrates such as glucose and fructose have similar cariogenicity to sucrose (Frostell, 1973; Imfeld, 1977), and the results from the Turku study comparing the effects of sucrose and fructose were inconclusive.

Therefore, although some results support the concept that sucrose is more cariogenic than other sugars, its major role in the aetiology of caries is believed to be due to the fact that it is eaten more often and in greater quantity than other sugars (Rugg-Gunn, 1983).

In addition to fermentable carbohydrate, other constituents of a food may be important in modifying the plaque pH response (Edgar et al., 1975; Rugg-Gunn et al., 1978), and many studies have investigated the effectiveness of "protective factors" such as inorganic phosphates in the

diet. However, the results from human clinical trials have beeen equivocal (Stralfors, 1964; Ashley et al., 1974).

Factors such as food consistency and rate of clearance from the mouth, pH and buffering power, and method of consumption may also be important (Holloway, 1983) and the sequence in which carbohydrate and non-acidogenic foods are eaten (Rugg-Gunn, 1983) may be relevant in determining the acidic environment surrounding a tooth.

1.10. The Carious Process.

Caries occurs as a result of the prolonged exposure of the tooth surface to the end-products produced by the metabolism of plaque microorganisms. Therefore, the sites which commonly develop lesions are those which allow plaque retention.

During eruption, when the teeth are not yet in occlusion, plaque can accumulate on their surfaces. This results in frequent episodes of de- and remineralisation at a subclinical level. When the teeth reach occlusion, most of these active lesions tend to become inactive as the microbial deposits on the surfaces are regularly disturbed by sheering forces from chewing and saliva (Fejerskov & Thylstrup, 1986). The exceptions to this are the pit and fissure regions which remain more sheltered from these protective influences. When interproximal contacts are formed, the bacterial deposits are removed from the contact area, but an ideal site for plaque accumulation develops below the contact point, and this area may progress to lesion formation.

The earliest visible change seen on smooth surfaces is loss of transparency of the enamel resulting in an opaque chalky region or "white spot" lesion. In the early stages of lesion formation there is minimal damage to the outer surface of enamel, but considerable demineralisation below the surface. (Darling, 1956; Soni & Brudevold, 1959). At this stage the lesion can be divided histologically into four zones which are usually clearly distinguishable by light microscopy (Silverstone, 1973). At the ultrastructural level, the first alteration seen is a scattered destruction of individual apatite crystals both within enamel prisms and at their boundaries. As the dissolution progresses, there is broadening of the

intercrystalline spaces. Measurement of crystal sizes within the light microscopic zones of a lesion show that in the surface and dark zones, the crystal diameters are larger than in sound enamel. This suggests that recrystallisation has taken place and is evidence that the caries process involves remineralisation as well as demineralisation (Kidd & Joyston-Bechal, 1987). The incipient lesion may be reversed at this stage if effective preventive measures are implemented.

However, if the local environmental factors favouring demineralisation outweigh those of remineralisation for prolonged periods of time, the number of dissolved crystals increases, and the crystal arrangement becomes disorganised. The calcified tissue then becomes more porous, the surface zone of the lesion eventually breaks down and a cavity forms (Kidd & Joyston-Bechal, 1987). Plaque can then accumulate within the cavity, leading to further progression of the lesion.

Chemistry of the Carious Process.

The spatial arrangement of the atoms in enamel resembles the mineral hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$. However the crystals lack stoichiometry due to deficiences in the three primary constituents (ie Ca^{2+}, PO_4^{3-} and OH^-), and their replacement in the lattice by CO_3^{2-}, HPO_4^{2-} and trace elements. Some of these ions, particularly carbonate, are relatively easily released from enamel during demineralisation, and positions in the lattice where CO_3^{2-} ions are present are believed to be particularly vulnerable to the effects of acids (Featherstone et al., 1979). Other ions, for example fluoride, may be included in the apatite proper and are only released when the crystal dissolves (Nikiforuk, 1985).

During the first few years after eruption of a tooth, secondary maturation of enamel takes place. This may be considered to be the result of the on-going de- and remineralisation that takes place during the establishment of the tooth in the oral cavity. During this phase, mineral is deposited from the oral fluids into the fine fluid-filled pores in the enamel, and there is release of the more readily dissolved mineral components and uptake of fluoride (Larsen & Bruun, 1986).

The physico-chemical integrity of enamel in the oral environment is dependent on the composition and chemical behaviour of the surrounding plaque and salivary fluids. The main factors governing the stability of the enamel apatites in saliva are the pH and concentrations of calcium, phosphate and fluoride in solution.

A simplified model of the physico-chemical equilibrium between enamel (hydroxyapatite - HAP or fluorapatite - FAP) and the aqueous phase is:

$$Ca_{10}(PO_4)_6(OH)_2 \rightleftharpoons 10Ca^{2+} + 6PO_4^{3-} + 2OH^-$$
$$Ca_{10}(PO_4)_6F_2 \rightleftharpoons 10Ca^{2+} + 6PO_4^{3-} + 2F^-$$

The product of the activities of these ions in the liquid phase governs whether or not the apatites dissolve. At equilibrium, the ion activity product is referred to as the solubility product. If these figures are exceeded, the solution is supersaturated with respect to the ions, whilst if the activity product is less than the solubility product, the solution is undersaturated and the salt tends to dissolve. Under normal physiological conditions, saliva is supersaturated with respect to both HAP and FAP, although the levels are not high enough to initiate spontaneous precipitation of the salts. Resting plaque fluid may be more supersaturated with respect to calcium and phosphate (Larsen & Bruun, 1986).

An increase in hydrogen ion concentration in the fluid environment of a tooth results in a decrease in hydroxyl concentration and conversion of the phosphate ionic species as shown in the equation:

$$\begin{array}{l} Ca_{10}(PO_4)_6(OH)_2 \rightleftharpoons 10Ca^{2+} + 6PO_4^{3-} + 2OH^- \\ \qquad\qquad\qquad\qquad\quad H^+ \downarrow\uparrow \quad H^+ \downarrow\uparrow \\ \qquad\qquad\qquad\qquad\quad HPO_4^{2-} \quad H_2O \\ \qquad\qquad\qquad\qquad H^+ \downarrow\uparrow \\ \qquad\qquad\qquad\qquad\quad H_2PO_4^- \end{array}$$

This results in an increase in the solubility of the enamel apatites as there will be a shift of the above reaction to the right. The concentration of calcium and phosphate ions already present in the oral fluids determine the pH at which the aqueous phase is saturated with respect to enamel apatites. The pH at which saliva is exactly saturated with respect to apatite is often referred to as the "critical pH", and will depend on the

concentration of calcium and phosphate in the particular saliva. Clinical assessment shows that the critical pH varies between pH 5.2 and 5.5.

Due to differences in solubility products of the apatites, when saliva becomes undersaturated with respect to HAP, it is still supersaturated with respect to FAP. The pH at which saliva is exactly saturated with respect to FAP is around 4.5. If the pH of the fluids surrounding the enamel is under 4.5, as may occur following the consumption of acidic fruits or beverages, the environment is undersaturated with respect to both apatites and therefore the enamel tends to be etched away leaving an erosion (Larsen, 1973).

However, in the pH range 5.5 - 4.5, as may occur in plaque fluid in situ following bacterial metabolism of carbohydrate, the fluid is undersaturated with respect to HAP and supersaturated with respect to FAP. This results in a deposition of FAP at the same time as HAP is dissolving. As the pH rises again, the aqueous environment of the enamel surface gradually returns to a state of supersaturation with respect to both apatites and this induces reprecipitation of mineral in the damaged area. However, as some ions may have diffused away from the subsurface enamel and inner plaque layer, they would be unavailable for redeposition, and the net result may therefore be a loss of mineral from the enamel.

During the very early stages of demineralisation there is a dissolution of the outermost layer of enamel at the ultrastructural level. Numerous tiny pathways between prisms and between crystals are opened up allowing acid to diffuse in, down a concentration gradient. As the acid diffuses in, its migration is limited as it reacts with enamel minerals (Larsen & Bruun, 1986).

The metabolism of carbohydrate by plaque bacteria results in the production of a variety of organic acids, as discussed in Section 1.8. An equilibrium exists beween the unionised form of these acids and the respective anion and hydrogen ion: $HA \rightleftharpoons H^+ + A^-$, with the equilibrium governed in each case by the dissociation constant, K_a for the acid.

Featherstone and co-workers (1979) have suggested that the unionised form is the predominant species of the acid which diffuses into the enamel via

fluid- filled pores. As it diffuses in, it dissociates partially into hydrogen ions and acid anions, both of which can attack the enamel crystal. The depth which the unionised species reaches is dependent on the rate of acid dissociation, which is in turn determined by the pH of the internal enamel fluids and the dissociation constant of the acid. It is therefore proposed that, in addition to lactic acid, high pK_a acids such as acetic acid play an important role in the carious process (Featherstone & Rodgers, 1981), and that the loss of acetic acid frequently observed around the pH minimum of a Stephan Curve is due to its diffusion into enamel in its undissociated form (Geddes et al., 1984).

The diffusion of acids through the outer layers of enamel in their unionised form, and their subsequent dissociation in the deeper layers away from the low pH of the acid-forming plaque surface, is one mechanism that has been proposed to explain the presence of the relatively intact surface layer found in early enamel lesions. A number of mechanisms have been put forward in an attempt to describe this phenomenon (reviewed by Arends & Christoffersen, 1986). The proposed mechanisms, involving the mineral rich outer layer of sound enamel, the organic matrix, the pellicle and the concentration gradients of ions, including fluoride and carbonate, across the enamel have all been shown to be inessential for surface layer formation, although they may influence the rate of its formation. Other models suggest the importance of the presence of adsorbed surface protective agents such as fluorides, salivary proteins and polyphosphates (Gray, 1977), or propose that dissolution-precipitation mechanisms operate, with the partially dissolved surface layer being continuously regenerated by calcium and phosphate ions dissolved from the subsurface enamel (Moreno & Zahradnik, 1974; Featherstone et al., 1978).

Arends and Christoffersen (1986), have recently proposed a new model which takes into account the fact that very early lesions formed in vivo do not have a surface layer, but only develop this mineral-rich layer as the lesions progress. They suggest that inhibitors such as fluoride ions and proteins play an important role in subsurface lesion formation. Following initial surface demineralisation, an influx of fluoride ions takes place from saliva and plaque into the liquid phase of the porous enamel. The concentration of fluoride in the aqueous phase decreases from the surface enamel in the direction of the lesion front. Adsorption of

fluoride ions at vacant OH^- sites on crystallites takes place near the surface, resulting in their protection from acid attack. Deeper in the lesion, where the fluoride concentration is lower, the aqueous phase remains undersaturated with respect to mineral, and so dissolution occurs. The formation of the mineral-rich surface layer is therefore influenced by the depth to which the fluoride ions can penetrate the lesion and the pH of the aqueous phase within the lesion.

1.11. Effect of Fluoride on Caries.

1.11.1. Historical Review.

At the beginning of the 20th century it became apparent that an association existed between staining of the teeth, described as enamel mottling, and some unknown factor present in the local environment. Analysis of water supplies for trace elements in the early 1930s found high levels of fluoride present in samples obtained from communities affected by this condition (Churchill, 1931), and it was therefore postulated that this agent was responsible for the observed alterations in the enamel.

Prior to this finding, a number of reports had discussed the possibility of a relationship between the mottling, or dental fluorosis, and dental caries (McKay, 1916 a & b; Ainsworth, 1928; Bunting et al., 1928). Studies were therefore designed to determine whether a concentration of fluoride existed which was low enough to prevent fluorosis, but high enough to have a cariostatic effect (Dean, 1945; 1954), and data obtained from these and other surveys suggested that a reduction of up to 50 % in caries prevalence, with no fluorosis of aesthetic significance, was associated with a concentration of one part per million (ppm) fluoride in the drinking water.

Originally the cariostatic effects were believed to be due solely to pre-eruptive influences, with the incorporation of fluoride into enamel taking place during mineral formation, but later studies observing children who had moved from communities with very low concentrations of fluoride in their water supply to areas with higher levels, found that a protective effect was obtained for teeth which were erupting or had newly erupted at the time of moving (Klein, 1946). In addition, observations of the caries

history of children moving from areas of high fluoride concentration to areas with low levels in their water supply, found that the cariostatic effects of fluoride were gradually lost (Russell, 1949). It therefore became apparent that the caries-reducing effect was dependent on continued exposure to fluoridated water supplies and that constant or periodic availability of fluoride to incipient lesions was essential for the maintenance of maximum caries protection.

1.11.2. Mechanisms of Action of Fluoride.

Fluoride plays a major role in the caries process due to its effects on the development and reactivity of the mineral phase of enamel, and its antimicrobial action on the plaque flora.

The presence of high levels of fluoride in the tissue fluids during mineral formation is believed to increase the stability of the apatite crystals formed, and hence reduce the solubility of the enamel. The proposed mechanisms by which this is achieved are:

i] Fluoride may act as a catalyst during mineralisation encouraging the transformation of soluble calcium-phosphate precursor phases into the more stable hydroxyapatite and fluorapatite forms (Amjad & Nancollas, 1979).

ii] The presence of fluoride in the tissue fluids may reduce the number of carbonate ions incorporated into the apatite (Nikiforuk et al., 1962), and hence reduce the solubility and reactivity of the enamel.

iii] The substitution of fluoride ions for hydroxyl groups in the lattice results in improved crystallinity of the apatite, with the formation of larger, less reactive crystals and reduced solubility of the enamel.

In addition to these ultrastructural effects, studies have shown that the consumption of approximately 1 ppm fluoride per day during tooth development may result in the formation of premolars and molars with less deep and narrow fissures (Jenkins, 1970), and it has been suggested that this may reduce the susceptibility of these sites to caries. However the

clinical significance of these findings is unclear, and this mechanism does not account for the observed reduction in smooth surface caries.

Until recently, the principal mechanism for the cariostatic action of fluoride was thought to be the replacement of hydroxyl ions by fluoride in the crystal lattice, thus making it less soluble in acid. However, even when fluoride is present in high concentration, only one tenth of the OH^- ions are replaced by fluoride (Larsen & Bruun, 1986). In addition, a concentration gradient exists across the enamel, so that although the outer 10 - 50 μm of recently erupted permanent tooth enamel may have a concentration of approximately 3,000 ppm fluoride, the enamel as a whole has a much lower level of 200 - 300 ppm; only 100 ppm higher than that found in teeth from individuals in non-fluoridated areas (Mellberg, 1977; Weatherell et al., 1977). The level of fluoride in subsurface enamel is almost always less than 500 ppm, and it is believed that at this concentration its solubility-reducing effect is minimal (Larsen & Bruun, 1986).

Current information therefore indicates that the incorporation of fluoride into enamel does not significantly influence the resistance of the tooth to the carious process (Weatherell et al., 1984). It is now believed that the most important tooth-protective function of fluoride is its ability to alter the saturation conditions in the oral fluids surrounding the enamel, as described in Section 1.10. The greatest benefits of these local, or topical, effects are derived shortly after the eruption of the tooth when microbial deposits tend to accumulate and frequent reductions in plaque pH occur. The acid attack results in preferential dissolution of crystals containing foreign ions such as carbonate, and the presence of fluoride in the surrounding fluids results in the earlier cessation of demineralisation as the pH begins to rise, and the formation of larger and more stable crystals which are more resistant to future acid attack.

Part of the cariostatic effect of fluoride is believed to be associated with the antimicrobial properties of this element (Hamilton, 1977). Fluoride exists in three forms in plaque; as strongly bound, loosely bound and free fluoride, and is present in much higher concentration than in saliva. Under acidic conditions, more free fluoride ions may be released from the loosely-bound fraction of plaque. The presence of extracellular fluoride

may interfere with the barrier function of bacterial cells, with a resultant disturbance in the gradients of ions on either side of the cell membrane and a depression in cellular activity. This effect is promoted by an acidic environment, and a reduction in pH may also increase the uptake of fluoride, in its protonated form, into the bacterial cell. Once in the cytoplasm, it dissociates again to F^- and H^+, with the increase in hydrogen ions causing a reduction in cellular metabolic activity, whilst the fluoride ions may inhibit glycolysis, at the enolase step, with the resultant reduction in the supply of phosphoenolpyruvate inhibiting sugar transport via the phosphoenolpyruvate-phosphotransferase system (Hamilton et al., 1985).

It has been suggested that the inhibition of carbohydrate metabolism by plaque bacteria may result in a shift in the microbial population to less acidophilic bacteria, but a study by Kilian and co-workers (1979b) found no significant change in the flora of plaque obtained from areas with high and low levels of fluoride.

The effect of fluoride on extracellular polysaccharides is uncertain. A reduction of one third of these polymers in plaque from subjects exposed to 1 ppm of fluoride was found in a study by Broukal and Zajicek (1974). Carlsson and co-workers (1969) have shown that glucosyl- and fructosyl-transferases are highly insensitive to fluoride, and it has been suggested that the observed reduction in the extracellular polysaccharides may be due to their enhanced degradation under the influence of fluoride (Schachtele, 1977).

1.12. Microorganisms Associated with Caries.

There is overwhelming evidence that the initial phase of dental caries involves demineralisation of tooth enamel by high concentrations of organic acids produced from metabolism of dietary carbohydrate by plaque bacteria accumulating on the tooth surface (van Houte, 1980). This concept is based on Miller's chemico-parasitic theory with the modification of dental plaque involvement introduced by Williams (1898) and Black (1898). Subsequent research which supports bacterial involvement in caries aetiology includes:

i] Studies demonstrating that the addition of penicillin to the diet of rats will prevent caries (McClure & Hewitt, 1946).

ii] Studies by Orland and co-workers (1954; 1955) with germ-free animals establishing the necessity of the presence of bacteria for the development of caries in rats.

iii] Studies by Keyes and Fitzgerald (Fitzgerald & Keyes, 1960; Keyes, 1960) showing that certain "caries-resistant" rodents differ from "caries-susceptible" ones in lacking a cariogenic flora, and that the former group will develop rampant decay when infected with appropriate bacteria.

The demonstration by Pasteur, Koch and other workers in the late 1800s that single microbial agents were the cause of different infectious diseases, initiated a search for a single causative agent of dental caries. Therefore, having shown a link between acid production by oral bacteria and enamel demineralisation, Miller then attempted to find one or several bacteria which were responsible for this acidogenesis. He found, however, that most, if not all, of the bacterial isolates he tested in vitro were capable of fermenting carbohydrate to low pH, and concluded that dental caries was bacteriologically non-specific and dependent on the accumulation of acid-producing bacteria for its initiation (reviewed by Loesche, 1982).

The relationship between plaque quantity and dental caries activity is however unclear. Some studies have shown that poor oral hygiene tends to correlate positively with caries increment or past caries experience (de Paola et al., 1968; Andlaw, 1978), and it is evident that caries occurs especially on areas of teeth where plaque accumulates most readily and in the highest amounts (van Houte, 1980). Conversely, other investigations have shown that many individuals remain caries-free despite unfavourable oral hygiene, or a tendency to form equal or even higher amounts of plaque than caries-active subjects (Mandel & Zengo, 1973).

One proposed explanation for this lack of clear-cut relationship between plaque mass and dental caries activity is that plaques vary in their microbial composition, and therfore in spite of being present in equal amounts, they may vary in their cariogenicity.

The frequent isolation of Bacillus (Lactobacillus) acidophilus from carious dentine (Howe & Hatch, 1917) and the ability of this organism to produce acid and artificial caries in in vitro models, led to a belief that this acidogenic and aciduric organism was the principal bacterial aetiological agent of caries (Kligler, 1916). However, as quantitative cultural techniques improved, it became apparent that lactobacilli were difficult to detect in plaque samples and were greatly outnumbered by other acidogenic organisms such as Streptococcus species. In 1924, Clarke isolated a streptococcus which occured more frequently than lactobacilli in early carious lesions. He named the organism Strep. mutans and showed that it was able to adhere to a tooth surface, and could produce artificial caries when incubated with teeth in a glucose broth. However, other researchers were unable to reproduce Clarke's data, and the potential cariogenicity of Strep. mutans remained largely neglected until the 1960s when intense investigation of this species was revived.

Conflicting opinions still exist as to whether only a small number of specific plaque organisms are responsible for the initiation of caries, or whether lesions can be produced by various combinations of the many acidogenic bacteria present in plaque. The two groups of organisms that have been specifically implicated in the aetiology of enamel caries are Strep. mutans and lactobacilli. The following sections will summarise the evidence advanced to support the role of these bacteria in caries initiation, and this will be followed by a discussion of the findings in favour of the non-specific hypothesis. In subsequent sections the studies investigating these theories will be described in greater detail, and their limitations discussed.

1.12.1. Streptococcus mutans and Caries.

The mutans streptococci are an antigenically and genetically heterogeneous group of non-motile, catalase-negative Gram positive cocci which characteristically ferment mannitol and usually sorbitol, in addition to other sugars (Mickalek & McGhee, 1982). They have been separated into six genotypes or subspecies (Coykendall, 1983; Beighton et al., 1984), five biotypes (Shklair & Keene, 1974; 1976) and eight serotypes (Bratthall, 1970; Perch et al., 1974 ; Beighton et al., 1981). It has been proposed that the six genotypes should be elevated to species level (Coykendall,

1983; Schleifer et al., 1984), and a brief summary of some of the biological features of the Strep. mutans group of bacteria is shown in Table 1. Strep. mutans (serotypes c/e/f) and Strep. sobrinus (serotypes d/g) are the species most commonly found in humans (Emilson & Thorselius, 1988), with serotype c strains being most frequently isolated, followed by d and e, whilst the others are only very rarely encountered (Hamada et al., 1976; Thomson et al., 1980; Carlsson et al., 1985). For the purposes of discussion in this thesis, the term Strep. mutans shall refer to the mutans group of streptococci as a whole, unless particular reference is otherwise made.

Ecological studies have shown that Strep. mutans colonises the mouths of infants only after tooth eruption and disappears from the mouth following the extraction of all the teeth (Nikiforuk, 1985). However its prevalence in edentulous denture wearers is similar to that found in dentate subjects (Emilson & Thorselius, 1988), thus supporting the theory that Strep. mutans is unable to persist in the oral cavity in the absence of a non-desquamating surface (Carlsson et al., 1969). The organism does not colonise the natural teeth uniformly, being isolated more frequently from fissures and interproximal surfaces than from buccal and lingual smooth surfaces. Studies using streptomycin-resistant mutants of Strep. mutans have shown that it does not readily spread from one tooth surface to another (Svanberg & Krasse, 1981). These findings may be due to its low salivary concentration and its relatively weak ability to adsorb to teeth (Gibbons & van Houte, 1975).

The term "The Specific Plaque Hypothesis" was introduced by Loesche (1976) to describe the concept that dental caries is a disease in which specific microorganisms play a major aetiological role. Many researchers believe that Strep. mutans is strongly associated with the initiation and development of human dental caries (reviewed by van Houte, 1980; Hamada & Slade, 1980; Loesche, 1982; Emilson & Krasse, 1985) and the following findings have been advanced to substantiate their argument:

i] Strep. mutans infection often precedes the development of caries (Ikeda et al., 1973; Köhler et al., 1981).

Genotype	I	II	III	IV	Unknown
Species	mutans	rattus	sobrinus	cricetus	ferus
Biotype	I & IV	II	IV & VI	III	unknown
Serotype	c e f	b	d g h	a	c
Fermentation					
Mannitol	+ + +	+	+ + +	+	+
Sorbitol	+ + +	+	± ± -	+	+
Raffinose	+ + +	+	- - -	+	-
Melibiose	+ ± +	+	- - +	+	unknown
Ammonia from arginine	- - -	+	- - -	-	-
Growth in bacitracin	+ + +	+	+ + -	-	-
Guanosine & cytosine mole %	36 - 38	41 - 43	44 - 46	42 - 44	43 - 45
Cell wall carbohydrate	Glucose Rhamnose	Galactose Rhamnose	Glucose Galactose Rhamnose	Glucose Galactose Rhamnose	Glucose Rhamnose

Table 1. Summary of some of the differences within the Streptococcus mutans group. From Russell (1987).

ii] The organism is isolated in higher numbers from carious lesions than from sound surfaces (Hoerman et al., 1972; Duchin & van Houte, 1978).

iii] A correlation is often found between caries prevalence and incidence and Strep. mutans levels in both plaque and saliva (Loesche et al., 1975; Zickert et al., 1982; Alaluusua & Renkonen, 1983).

iv] Correlations often exist between the progression of carious lesions and Strep. mutans levels (Zickert et al., 1982).

v] Strep. mutans produces high levels of multi-surface caries in animals, in contrast with other species of dental bacteria which are less cariogenic (Hamada & Slade, 1980).

The pathogenicity of Strep. mutans has also been extensively studied (reviewed by Hamada & Slade, 1980; van Houte, 1980; Bowden et al., 1984; Emilson & Krasse, 1985) and some of its biochemical properties which are believed to be of importance in relation to the initiation and development of caries include:

i] The production from sucrose of extracellular polysaccharides which may mediate its adherence to tooth surfaces (Hamada & Slade, 1980).

ii] The formation of intracellular polysaccharide energy reserves (Emilson & Krasse, 1985).

iii] The rapid metabolism of sugars to organic acids, particularly lactic acid (Minah & Loesche, 1977; van der Hoeven & Franken, 1982).

iv] The ability to initiate and maintain microbial growth and to continue acid production at low pH levels (Harper & Loesche, 1984).

1.12.2. Lactobacilli and Caries.

The lactobacilli are a group of non-sporing, usually non-motile, catalase negative Gram positive bacilli of variable length (Koneman et al., 1983). The genus is divided into two groups dependent on the products formed from glucose metabolism, with those producing more than 65 % lactic acid being classified as homofermentative, and those forming less than 65 %

lactic acid and significant amounts of carbon dioxide, acetic acid and ethanol, being referred to as heterofermentative (Hardie, 1983). The homofermentative species are L. acidophilus, L. salivarius, L. casei and L. plantarum, and species belonging to the heterofermentative group include L. fermentum and L. brevis.

Lactobacillus spp. have been implicated in the aetiology of dental caries for many decades, and some of the evidence which has been put forward to support its role in relation to caries development is as follows:

i] Lactobacillus spp. are found, often in high numbers, in nearly all carious cavities (reviewed by van Houte, 1980).

ii] Their proportions in plaque and saliva are often positively correlated with caries activity (Loesche & Syed, 1973; Burnett et al., 1976).

iii] Lactobacillus spp. have a high acid production rate and are extremely aciduric organisms, being able to initiate and maintain growth at low pH levels, and produce lactic acid in conditions below pH 5.0 (Harper & Loesche, 1984).

iv] Some strains of lactobacilli are able to produce caries in gnotobiotic rats, although not to the same extent as Strep. mutans (reviewed by Loesche, 1982).

v] Extra- and intracellular polysaccharides can be synthesised from dietary sucrose by certain lactobacillus species (reviewed by Edwardsson, 1986).

Lactobacillus spp. have a relatively low affinity for the tooth surface (van Houte et al., 1972) and surface defects such as carious lesions may favour the retention of this organism. In addition, the low pH of these sites will also select for these aciduric bacteria, allowing them to multiply in the acidic conditions within a lesion.

Lactobacillus spp. are only rarely isolated from plaque prior to the development of caries, and are often absent from plaque overlying incipient lesions. Some workers have therefore argued that the presence of lactobacilli in a carious lesion is an opportunistic proliferation rather than a causal event (Johnson & Colman, 1986), and that these organisms may

not be involved in the initiation of caries, but may contribute to the progression of already existing lesions (reviewed by Loesche, 1982).

A number of experimental studies have shown a positive correlation between the number of lactobacilli in saliva and the intake of dietary carbohydrate (Jay, 1947; Featherstone, 1960). Salivary lactobacillus counts have therefore been used to monitor the effectiveness of dietary counselling (reviewed by Karjalainen, et al., 1987) with some studies showing an alteration in the level of this organism associated with changes in the intake of dietary sugars (reviewed by Stecksén-Blicks, 1987). Others however, have been unable to demonstrate a positive correlation (Krasse, 1954) or have only shown a weak association between these two parameters (Crossner, 1984; Stecksén-Blicks, 1987). Also, it has been suggested that other factors in the oral cavity such as increased microbial retention sites and reduced salivary flow rate and buffering capacity (Parvinen & Larmas, 1981; Heintze, 1984) may also be closely related to high lactobacillus counts.

1.12.3. Relationship of Other Plaque Organisms to Caries.

Plaque bacteria, other than Strep. mutans and lactobacilli, have been studied less thoroughly for their relationship with dental caries, (reviewed by van Houte, 1980; Edwardsson, 1986), and the results obtained have, in many cases, been equivocal.

Strep. sanguis and Strep. mitis are prominent members of the plaque flora, are acidogenic, and some strains have the ability to produce intra- and extracellular polysaccharides and to create enamel lesions in gnotobiotes (reviewed by Edwardsson, 1986). However, in vitro studies have shown they produce less acid and are less aciduric than Strep. mutans (reviewed by Loesche, 1982), create fewer and less severe lesions in animal models (van Houte, 1980), and human studies have failed to find a positive relationship between Strep. sanguis or Strep. mitis and caries activity (Bowden et al., 1976; Dreizen & Brown, 1976).

Actinomyces species are also present in large numbers in mature plaque and are often found in plaque overlying caries lesions (Loesche & Syed, 1973). However, little data exists regarding their association with the

initiation of caries, but the information that is available suggests they do not play a major role in the aetiology of enamel caries (Bowden et al., 1976; Hardie et al., 1977; Minah & Loesche, 1977), although they have been implicated in relation to the initiation of root surface caries (Syed et al., 1975).

Candida species generally constitute only a small fraction of the plaque flora (Theilade et al., 1974). In vitro studies have shown that they are acidogenic and are able to survive and metabolise carbohydrate at pHs in the vicinity of 5.0 (Loesche, 1982). They have been isolated from carious dentine (Hodson & Craig, 1972) and some studies have shown an association between salivary levels of candida and caries prevalence (Banoczy, 1983). It is probable that the relationship reflects the ability of candida to colonise the low pH environment surrounding a carious lesion, although the association between candida and caries development remains unclear.

Veillonella species are the most prominent Gram negative species present in supragingival plaque samples. They are unable to ferment common dietary carbohydrate, but can utilise organic acids, such as lactic acid, as an energy source. The metabolism of lactic acid to weaker acids such as acetic, propionic and formic (Distler & Kröncke, 1980) may reduce plaque acidity and therefore modify the cariogenic potential of the microflora. Attempts to correlate veillonella and caries activity have produced conflicting results, with a positive correlation being noted in one study (Mazzarella & Shklair, 1960) but not in others (Sims & Snyder, 1958; Minah & Loesche, 1977). The presence of this organism may reflect the high acidity and cariogenic potential of an environment, or the organism may metabolise the lactic acid to such an extent that it reduces the cariogenic potential and thus selects for less aciduric organisms (Loesche, 1982). A study by Mikx and co-workers (1972) showed that the addition of veillonella to a Strep. mutans flora substantially reduced the incidence of caries in gnotobiotic rats.

Although strong evidence exists which suggests that Strep. mutans and lactobacilli are often associated with the initiation and development of caries, and Strep. mutans appears to possess all the biochemical characteristics believed to be of importance in the pathogenicity of this disease (Loesche, 1982), other plaque organisms also possess some of these

cariogenic properties, and will play a role in the development of lesions. Individual species, or combinations of organisms, may possess the necessary characteristics to enable them to produce lesions in the absence of Strep. mutans and lactobacilli, and some of the evidence supporting "The Non-specific Plaque Hypothesis" is summarised below:

i] Many oral bacteria including streptococci, lactobacilli, actinomyces and yeasts are acidogenic and are found in relatively high numbers in the oral cavity.

ii] Comparison of plaque from caries-active and caries-inactive patients has shown that the total acidogenic numbers of plaque organisms is higher in the former group (Edwardsson, 1986).

iii] In addition to Strep. mutans, many other oral bacteria including Strep. sanguis, Strep. mitis, A. viscosus and A. naeslundii are also able to produce extracellular polysaccharides, to varying extents, in the presence of sucrose (Minah & Loesche, 1977).

iv] Several groups of organisms in dental plaque such as Strep. mutans, Strep. mitis, Strep. sanguis, A. viscosus and L. casei have the ability to produce intracellular polysaccharides (Gibbons & Socransky, 1962). Endogenous metabolism of these polymers would maintain acid production in plaque for long periods in the absence of exogenous sugar sources.

v] In addition to Strep. mutans and lactobacilli, many other plaque organisms including some strains of Strep. sanguis, Strep. salivarius, Strep. mitior, A. viscosus and A. naeslundii are capable of producing caries, to various extents, in gnotobiotic rats.

1.12.4. Cariogenicity Models.

The complexity of the bacterial community found on enamel surfaces, the difficulties involved in accurate removal and identification of organisms from carious and caries-free sites, the problems associated with diagnosis of early lesions, and the fact that many other factors including enamel susceptibility, dietary and salivary characteristics also play a major role in the aetiology of caries, makes it extremely difficult to study the relationship of bacteria to the initiation of caries and, in particular, to attempt to implicate specific groups of organisms in its aetiology.

However, since the appropriate treatment and preventive measures adopted to deal with this disease may be influenced by whether the non-specific or specific concept is followed, extensive research has been undertaken in an attempt to resolve this problem but at present the results remain equivocal. These studies have involved i] in vitro investigations, ii] animal models, and iii] human epidemiological and experimental studies.

1.12.5. In vitro Studies.

In addition to whether a bacterium is present in high numbers, the contribution of the different plaque organisms to caries initiation may also be dependent on the extent of its acid production at various pH levels and on the lowest pH at which it can ferment carbohydrate and remain viable.

The results obtained from in vitro studies may be used to support both the non-specific and specific hypotheses, since they show that many oral bacteria are able to produce acids from carbohydrate (Ellen & Onose, 1978; van Houte, 1980), but that some strains of Strep. mutans and lactobacilli are more acid tolerant than a variety of other plaque species such as Strep. sanguis, Strep. salivarius and Actinomyces viscosus (Harper & Loesche, 1984), being able to initiate and maintain plaque growth and to continue to produce lactic acid at low pH levels.

1.12.6. Animal Models.

Following the development of the animal models which demonstrated the absolute requirement of bacteria in the aetiology of caries (described in Section 1.12.), these systems, using gnotobiotic and conventional rodents, were used to determine the cariogenic potential of different oral bacteria (reviewed by van Houte, 1980). It was found that not all acid-producing organisms were able to induce caries, and that some species could produce demineralisation only on specific sites on a tooth surface. It therefore became apparent that in addition to its acidogenic potential, an organism's ability to adhere to surfaces and to compete with other members of the microbial population may play a significant role in determining its demineralising potential. Strains of Strep. mutans were almost always found to be cariogenic in rats fed a high sucrose diet, whilst other streptococci, lactobacilli and actinomyces were more variable in their

cariogenicity. The spectrum of organisms capable of producing demineralisation was smaller in hamsters than in rats (reviewed by Rosen, 1984). Generally the caries induced by these other organisms was much less severe than with Strep. mutans and was virtually exclusively restricted to the tooth fissures (van Houte, 1980).

Animal models have also allowed variations in the microbial traits of bacterial species to be studied in an attempt to discover which are important in relation to cariogenicity; most investigations have been conducted with Strep. mutans. Enzyme-deficient mutants have shown that reduced acidogenic potential due to defective lactate dehydrogenase (Hillman, 1978) and diminished extracellular and intracellular polymer producing capabilities (Freedman et al., 1979; Tanzer et al., 1976) have resulted in reduced caries experience.

Studies on microbial interactions involving the inoculation of more than one bacterial type into gnotobiotic animals have shown that some combinations may produce higher caries rates than those found when the organisms are inoculated separately, whilst in other cases the interaction between bacteria may reduce the cariogenic challenge; for example the lactic acid produced by Strep. mutans may be converted into weaker organic acids by Veillonella species (reviewed by Edwardsson, 1986).

Since the thickness and mineral component of monkey enamel more closely resembles that of humans than does rodent enamel, conditions found in humans are better simulated in monkey models. Longitudinal investigations comparing the caries activity of Strep. mutans infected monkeys and uninoculated controls, both on high cariogenic diets, have found much higher levels of caries in the Strep. mutans - inoculated animals (Bowen, 1969). The importance of Strep. mutans in caries aetiology has also been supported by immunisation experiments (Bowen et al., 1975; Lehner et al., 1977) which have shown a reduced incidence of caries in monkeys given Strep. mutans vaccines.

1.12.7. Human Studies.

Although much evidence can be gained from in vitro and animal studies, caution must be exercised in attempting to extrapolate the results of these

experiments to the human situation. Numerous epidemiological surveys have therefore been undertaken in an attempt to associate the presence of specific bacteria in dental plaque or saliva with the initiation of caries. Most of these studies have focused on streptococci and lactobacilli and have been conducted in either a cross-sectional or longitudinal manner (reviewed by Mikkelsen et al., 1981; Loesche et al., 1984; Marsh & Martin, 1984; Boyar & Bowden, 1985; Edwardsson, 1986).

Several cross-sectional studies have revealed a positive correlation between the presence and levels of Strep. mutans, from either salivary or plaque samples, and the prevalence of dental caries (Loesche et al., 1975; Klock & Krasse, 1977) and some have shown an association between salivary lactobacillus counts and the presence of carious lesions (Klock & Krasse, 1977; Zickert et al., 1982). However these studies do not allow a cause-and-effect relationship to be established, since these bacteria may colonise or become prominent within the community only once a lesion has been initiated, as a response to changes in environmental conditions.

Longitudinal epidemiological surveys enable more accurate assessment to be made of the relationship of specific bacterial populations to the initiation of caries. Here plaque sampling from, and examination of, initially sound tooth surfaces are carried out at regular intervals, allowing the development of caries to be related to the levels of particular species present just before the clinical detection of the lesion. However, longitudinal studies are very time-consuming, labour intensive and expensive, being limited by the lengthy course of the disease, and the problems associated with the detection of early caries. Large numbers of teeth have to be included in the study, and difficulties are experienced in accurate and repeated plaque sampling from discrete sites without contamination from adjacent areas. Lengthy and expensive microbiological procedures are involved in the identification of the plaque isolates. As a result, only a relatively small number of longitudinal studies have been carried out, and the results obtained relating the development of caries with specific organisms have been inconclusive.

Although many of the longitudinal studies support a role for Strep. mutans in the development of caries (reviewed by Loesche et al., 1984), and in some cases an increase in the plaque levels of Strep. mutans has

been found shortly before caries detection (Ikeda et al., 1973; Köhler et al.,1981), most have shown that the increase of this species took place either at the time of caries diagnosis or at a later stage in the development of the lesion (Hardie et al., 1977; Loesche & Straffon, 1979). Some lesions developed in the presence of only low levels of Strep. mutans (Masuda et al., 1979) or in the absence of detectable levels of this organism (Hardie et al., 1977; Loesche & Straffon, 1979) whilst in other cases sites remained caries-free despite being covered by plaque containing high levels of Strep. mutans (Loesche & Straffon, 1979; Loesche et al., 1984). An increase in plaque lactobacilli has been observed at the time of caries diagnosis (Ikeda et al., 1973; Loesche & Straffon, 1979) and has been associated with the progression of lesions through the enamel (Hardie et al., 1977; Boyar & Bowden, 1985). This change in lactobacillary levels has been interpreted as being secondary to the appearance of the clinical lesion (Ikeda et al., 1973; Loesche, 1982), but in some cases the levels of this organism have also been found to increase 6-12 months prior to the diagnosis of caries (Loesche et al., 1984). These findings, together with the fact that the organisms are present in such low proportions in plaque, make interpretation of the role of lactobacilli in the initiation and development of caries extremely difficult.

Attempts have been made to overcome some of the problems associated with longitudinal epidemiological studies by designing models for the human oral cavity which encourage lesion formation, and allow better detection and quantification of demineralisation and more accurate plaque sampling. Incipient lesions have been produced within three weeks by promoting cariogenic conditions in localised areas of the dentition of volunteers (Scheie et al., 1984) and although such lesions are considered to be reversible, in an attempt to overcome ethical difficulties, some studies have been performed using teeth which are later to be extracted for orthodontic purposes. Orthodontic bands or gold plates are attached to these teeth, leaving stagnation ares which encourage proliferation of plaque organisms at these sites (Ögaard et al., 1984; Arneberg et al 1984; Holmen et al., 1985). The teeth can be sectioned following their extraction, allowing quantification of the demineralisation.

Instead of using the volunteer's own dentition, other intra-oral models have examined the effect of the flora on enamel obtained from exogenous

sources. Fissure inserts, using the crowns of unerupted teeth, may be implanted into the volunteer's occlusal restorations (Theilade et al., 1974) or into an acrylic appliance (Thott et al., 1974; Minah et al., 1984; Strassler et al., 1986). However, problems are encountered with plaque sampling since pooled samples are usually collected from the whole of the fissure and discrete sampling of the carious site is not possible. Ethical difficulties again arise in attempting to create cariogenic conditions in these selected sites without affecting the volunteer's own dentition.

Enamel slabs may also be mounted on to intra-oral appliances, and the first use of this type of model was made by Koulourides and co-workers in 1974, to study the in situ remineralisation of enamel lesions. Since then many other remineralisation studies have been carried out using a similar model (Mellberg & Chomicki, 1983; ten Cate & Rempt, 1986; Dijkman et al., 1986). Appliance models have also been used in cariogenicity tests. Most have been employed to assess the cariogenic potential of various sugars, and have involved enamel slabs, mounted on the appliance, with or without a coating of Strep. mutans cells on the surface (Koulourides et al., 1976; Tehrani et al., 1983; Brudevold et al., 1984; Tehrani et al., 1986).

Only a few demineralisation studies using an appliance model have examined the composition of the plaque flora overlying the enamel slabs, and those that have done so have identified only the main morphological groups present, and have covered the enamel surface with Dacron gauze to encourage the retention and proliferation of the plaque microflora (Ostrom et al., 1977; Gallagher & Pearce, 1979).

The use of enamel slabs normally allows quantification of the mineral content of the test site to be carried out only at the end of an experimental period, since this assessment often involves destruction of the slab. A slab of enamel from an adjacent site on the same tooth is used as a control. However, since the susceptibility of an enamel surface to a cariogenic challenge varies even between various sites on the same tooth (Groeneveld et al., 1975) more accurate assessment of the demineralising effect of an agent would be gained from using the same area of enamel as its own control. This can be achieved by the use of thin enamel sections, which allow accurate measurement of the mineral content of the enamel to be made both pre- and post-experimentally, and through-out the

test, without destroying the enamel. This "single section" technique has been used in in vitro (Harvey et al., 1982; Featherstone & Silverstone, 1982) and in situ (Creanor et al., 1986a) remineralisation investigations. A study by Creanor and co-workers (1986b) has shown that the mounting of enamel sections on recessed troughs on the fitting surface of acrylic lingual flanges of a lower removable appliance allows plaque to accumulate on the enamel surface without a gauze covering being required. In addition they have also shown that the microbial composition and acid anion profiles of the experimental plaque is similar to that obtained from the natural dentition.

1.13. Aims of the Study.

As outlined in Section 1.12., conflicting opinion still exists as to whether specific bacteria are the cause of dental caries, and many problems are associated with attempting to carry out studies designed to help to resolve this problem.

The initial aim of this study was to develop an in situ model, using enamel sections mounted on a lower removable intra-oral appliance to investigate the relationship between the composition of the plaque microflora and underlying enamel demineralisation.

The model would require to produce rapidly quantifiable demineralisation of enamel sites and allow accurate sampling of plaque from discrete test sites. A number of preliminary experiments were to be conducted to test the validity of the model involving:

i] Testing the reproducibility of the identification and enumeration of the plaque microflora,

ii] Comparing the composition of the microflora of plaque samples, obtained from enamel specimens mounted on the appliance, with the microbial composition of plaque from the subject's natural dentition and from the acrylic of the appliance,

iii] studying the variation in the susceptibility of enamel to demineralisation.

In the main appliance studies, the aim was to assess whether a relationship existed between the composition of plaque microflora and the quantity of mineral loss in underlying enamel produced under various test conditions, in seven subjects.

In addition to attempting to determine the relationship between the numbers of organisms present and demineralisation, an in vitro study was also carried out to determine whether variation existed in the cariogenic potential of strains of Strep. mutans isolated from the different volunteers, and if so, whether these findings correlated with the natural and experimental caries experience of the subjects.

The aim of the final part of the project was to determine whether the intra-oral appliance system, with its facility for obtaining absolute and proportional counts of microorganisms in plaque samples from discrete enamel sites after varying time intervals, would prove to be a useful model for studying the early colonisation of enamel and subsequent plaque development.

CHAPTER II

METHODS AND MATERIALS.

2.1. Introduction.

Both the main study, designed to examine the relationship of the plaque flora composition to enamel demineralisation, and the investigations of microbial colonisation and development of plaque on enamel surfaces, were performed intra-orally using a removable appliance.

In this chapter, the appliance design and construction is described, together with the methods involved in preparing the enamel sections and slabs, and in quantifying their mineral content. This is followed by a description of the techniques used to collect, culture and identify the plaque microflora from the test sites and to carry out acid anion estimations and plaque pH measurements following a sucrose challenge. A description of preliminary studies, which were carried out during the development stage of the in situ model, using the methods described below, follows in Chapter III.

2.2. Appliance design.

The lower removable appliance, as described by Creanor and co-workers (1986a), was employed in this study. However, a cobalt-chromium framework, with lingual plate and clasps, was used instead of the previously described all acrylic design as the former improved retention and was more comfortable for the volunteers (Fig. 2.1.).

The stages involved in the construction of an appliance were as follows;- upper and lower impressions were taken in alginate (Xantalgin, Bayer, Newbury, England) and from these, models were poured in dental stone. Undercut areas present on the lower cast were blocked out with plaster and the cobalt-chromium framework was constructed with clasps in the $\overline{65}|\overline{56}$ regions. This was then inserted into the volunteer's mouth and any necessary adjustments made. The cast then had pieces of wax (dimension 16 x 9 x 1 mm) added to the lingual lower first and second molar regions

on either side. The model was then duplicated and acrylic lingual flanges added to the appliance framework. In this way recessed troughs were incorporated into the fitting surface of the flanges, the dimensions corresponding to that of the wax templates. An entrance channel was cut in the centre of the acrylic upper edge of the trough area (Fig. 2.2.) and the lower edge was opened to allow a free flow of saliva through the site. Enamel sections and slabs could be mounted in these recessed sites without contacting the natural tooth surface or lingual mucosa.

2.3. Tooth Preparation.

2.3.1. Source and Examination of Teeth.

Many of the methods used in the preparation of enamel sections were based on the techniques described by Creanor (1987), with appropriate modifications for use in this demineralisation study.

Human premolar teeth, extracted for orthodontic purposes, were obtained from the Glasgow area (water fluoride < 0.03 ppm), and following storage in a 5 % aqueous thymol solution they were cleaned with a fine pumice-alcohol mixture to remove any pellicle. The crowns were then washed in warm soapy water, dried in air, and the buccal and lingual surfaces of the crown examined using a dissecting microscope at 10 x magnification. Only teeth with no obvious flaws or incipient carious lesions were selected for use in the study.

2.3.2. Preparation of Sections.

The crown of the tooth was removed using a dental drill (Milbro, Epson, England) and diamond disc (diameter 2.5 cm), leaving approximately 1-2 mm of root just apical to the amelo-cemental junction. The crown was then halved mesio-distally and each half mounted on to an acrylic block using "Loc-tite" adhesive (Loctite (U.K.) Ltd., Welwyn Garden City, England).

Following hardening of the adhesive, the acrylic blocks were fitted into the chuck of a Leitz saw microtome (Luton, England; Fig. 2.3.).

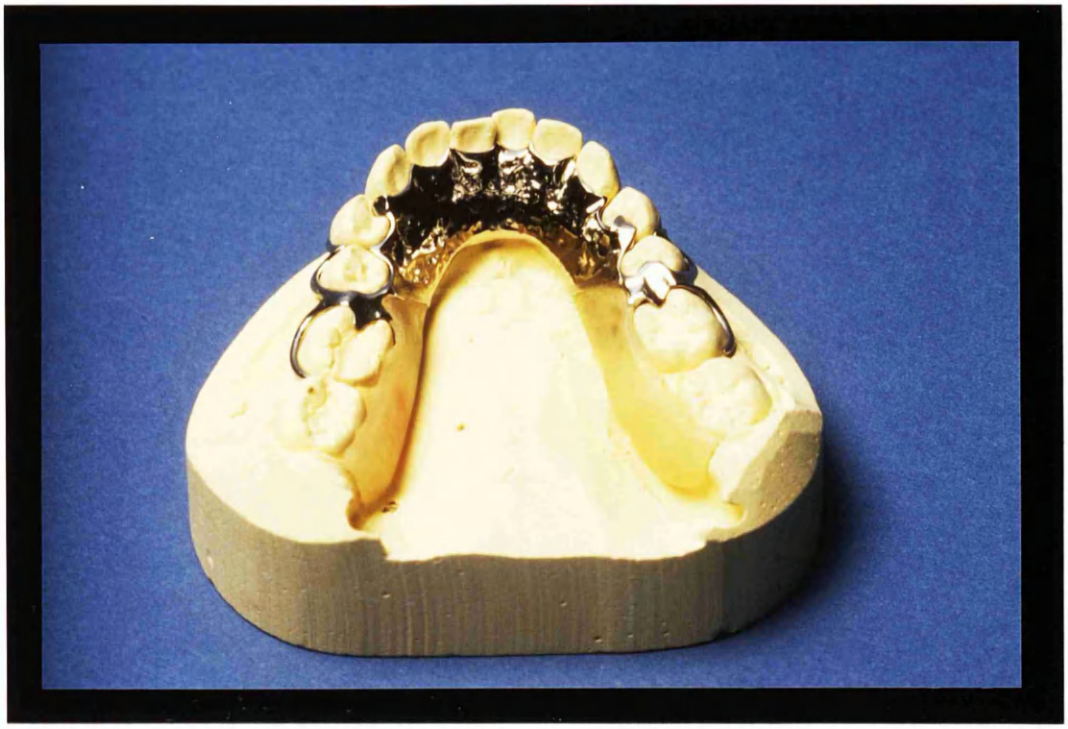


Fig. 2.1. Lower removable appliance, mounted on study cast.

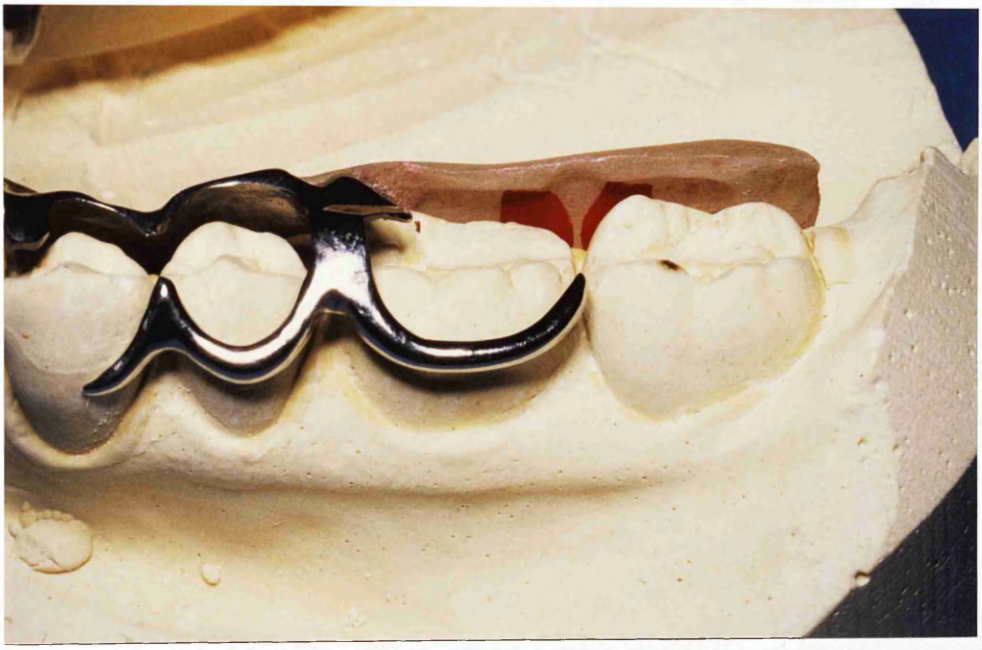


Fig. 2.2. Close-up of upper edge of lingual flange, showing central entrance channel.

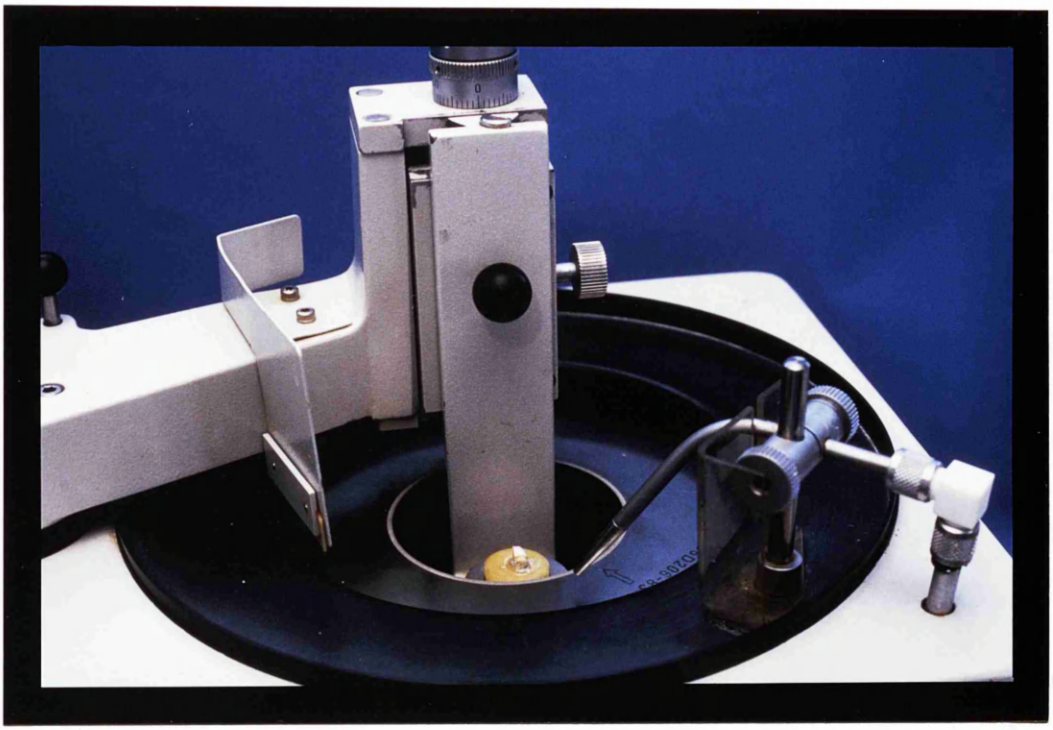


Fig. 2.3. **Saw microtome with tooth, mounted on acrylic block, in position.**

Longitudinal sections of the teeth were then cut to an approximate thickness of 250 μm using the slowest approach speed setting of the microtome. Each section was coded using a graphite pencil with a letter representing the tooth and a number corresponding to the sequence in which the section was cut. The sections prepared from the buccal portion of an individual tooth were used for an experimental run in one subject with alternate sections being mounted on opposite sides of the appliance. The lingual portion of the same tooth was used to prepare sections for a second volunteer.

2.3.3. Section Grinding.

A slurry of aluminium oxide powder (Raymond A. Lamb, London, England) and water was mixed on a ground glass plate (Shandon Southern Products, Runcorn, Cheshire, England; Fig. 2.4.). Specimens were placed on a heavy brass plate which had been covered with a damp gauze. The brass plate was then inverted and slowly rotated round the glass plate. The presence of the gauze increased the friction between the section and the moving surface ensuring that the hard tissue remained in contact with the gauze. In this way, the surface of the section in contact with the glass plate was ground. Each cut surface of the section was treated in this manner until the required thickness was attained, and immediately thereafter the identification code of the section was re-applied as described in Section 2.3.2.

2.3.4. Measurement of Section Thickness.

A digital micrometer (Mitutoyo, Tokyo, Japan; Fig. 2.5.), was used to measure the section thickness. The specimen was placed on the flat table of the instrument and a measuring probe gently lowered until it came into contact with the enamel portion of the section. Measurements of section thickness were made from four regions of the enamel on each specimen, thus allowing the planoparallelity as well as the thickness of the section to be assessed. Sections were prepared to a final thickness of approximately 100-120 μm and were then stored in sterile deionised water prior to use.

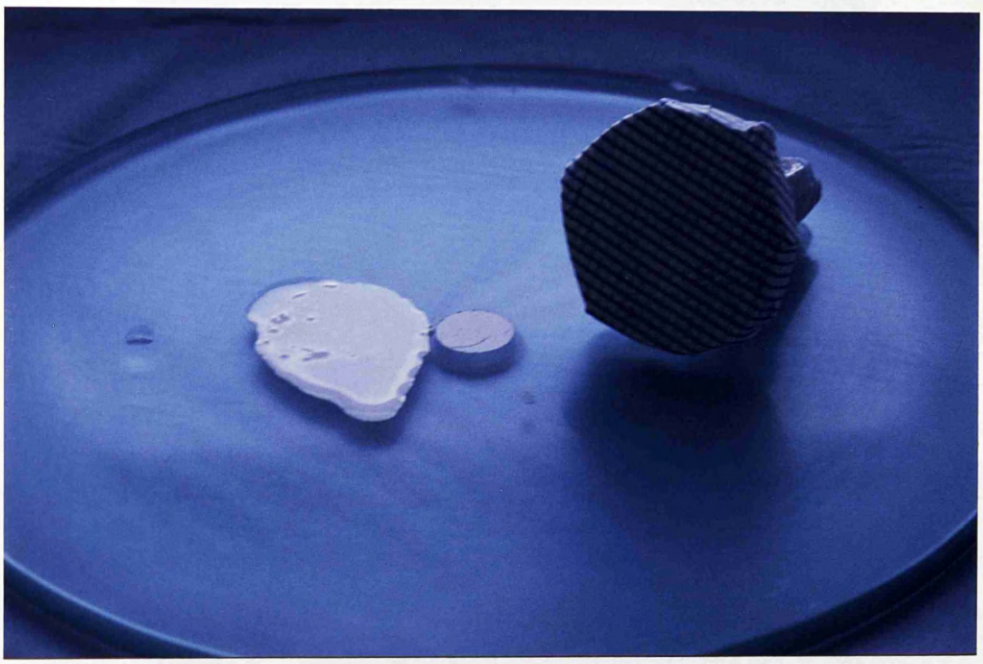


Fig. 2.4. **Brass plate with gauze covering and alumunium oxide slurry on ground glass plate.**

Fig. 2.5. **Digital micrometer used for measurement of enamel section thickness.**

2.3.5. Varnishing and Window Preparation.

All cut surfaces of the enamel sections were entirely covered with a coat of proprietary nail varnish (Max Factor, London, England). The natural enamel edge was also covered, except for two windows of approximately 1.5 - 2.0 mm in length on each section (Fig. 2.6.). The coated sections were then left for 2 - 3 hours to allow the varnish to set. The sections were then given a second coating of varnish and left for a further 2 - 3 hours before being mounted on the trough area of the appliance.

2.3.6. Mounting of Sections.

Two sections were mounted on each side of the appliance using nail varnish (Fig. 2.7.). They were placed vertically in the centre of the acrylic trough area with the natural enamel edges facing one another. The sections were positioned with a space of approximately 1 mm between them, to allow the plaque overlying each enamel window to be removed accurately at the end of the experimental period.

At the start of each experiment, a diagram of the position of the windows on the enamel sections, and of the sections on the trough area, was drawn to facilitate the localisation and identification of the test sites at the end of each wearing period.

2.3.7. Slab Preparation.

Enamel slabs were prepared from caries-free premolars which were washed as described in Section 2.3.1. The enamel face from each surface was removed using a dental drill and diamond disc, cutting just into the dentine and parallel to the surface of the tooth. The dentine surface of each face was then handground using the aluminium oxide slurry and ground glass plate. This produced a flat surface and a slab thickness of 400-600 μm. The face was then cut into slabs of approximately 4 - 7 mm^2. The cut surfaces of each slab were varnished, leaving the natural enamel surface uncovered. These slabs were then mounted on to the trough area of the appliance using the varnish. The positioning of the slabs in the recessed sites varied depending on the experiment being conducted, and these details will be described in the appropriate sections.

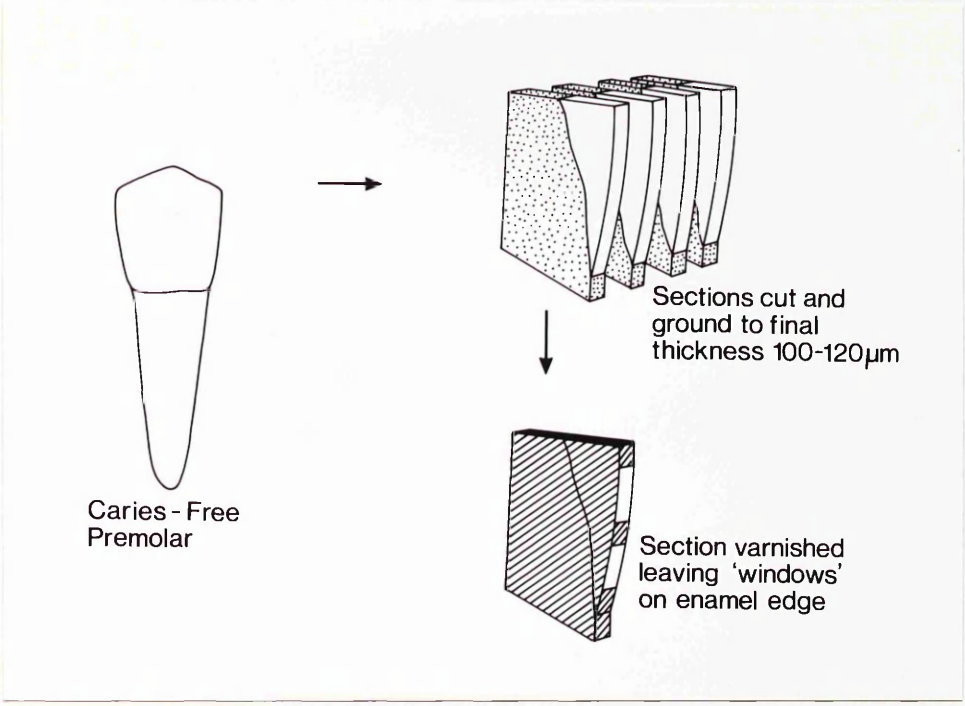


Fig. 2.6. **Diagram showing preparation of varnished enamel sections.**

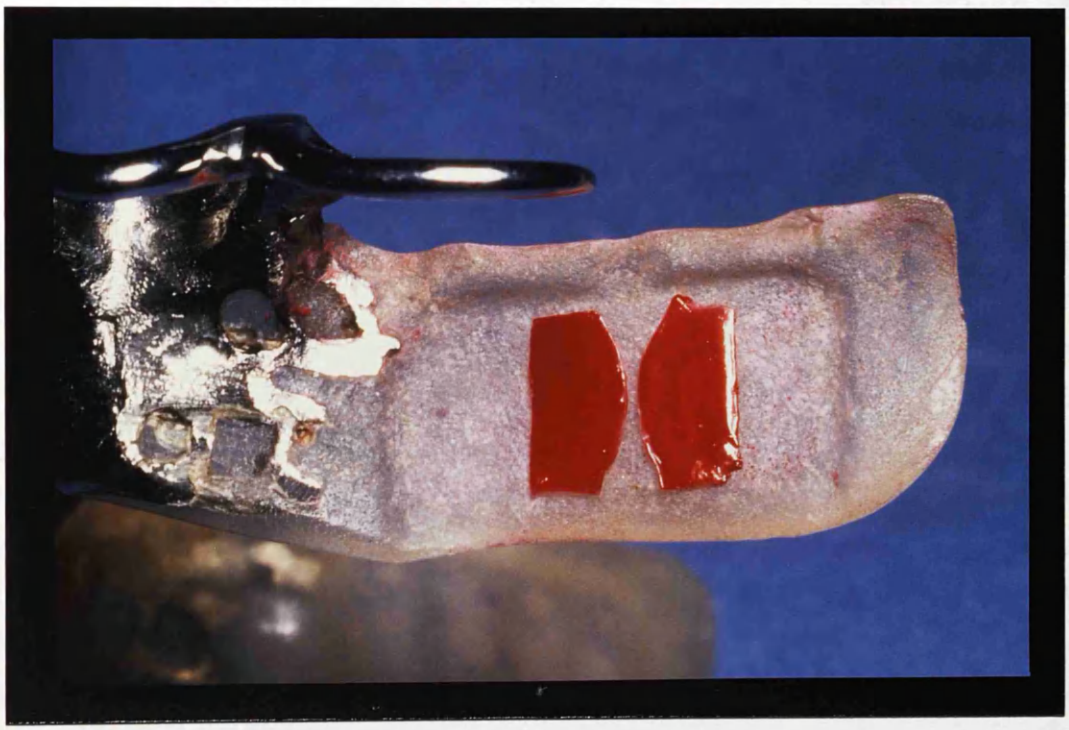


Fig. 2.7. **Varnished sections mounted on acrylic trough area of appliance.**

2.3.8. Measurement of Surface Area of Slabs.

The surface area of each slab was measured by computerised planimetry (Fig. 2.8.). A Leitz microscope with a drawing tube attachment (Luton, England) allowed a light spot on a probe to be projected into the microscope field while the probe was held in contact with a bit-pad (Video Vector Dynamics Ltd., Glasgow, Scotland). When the area of a slab was traced with the light spot of the probe, a corresponding area was traced on the bit-pad. The bit-pad was connected to a North Star Advantage graphics computer (Video Vectors Dynamics Ltd., Glasgow, Scotland) which provided a visual representation of the tracing and allowed the area of a slab to be expressed directly in units of mm^2. Three measurements were obtained for each slab, and the mean calculated.

2.4. Microradiography and Microdensitometry.

2.4.1. Microradiographic Methods.

In order to determine whether changes in the mineral content of the enamel windows had taken place during experiments, microradiography of the sections was carried out prior to mounting the specimens on the appliance, and then again at the end of each experimental period.

Enamel sections, of known thickness, together with an aluminium stepwedge (thickness ranging from 50 - 300 μm) were placed between two single layers of clingfilm (Fig. 2.9.). The clingfilm was then wound round a Kodak high resolution radiographic plate (Type 1A) (Eastman Kodak Company, Rochester, New York, USA), so that its contents were held in very close proximity to the plate with only a single layer of the plastic intervening. The plates were exposed to Cu Kα Ni filtered radiation from a Marconi X-ray tube (TX12) in an Enraf Nonius generator for 20 min at 20 kV and 30 mA at a target-source distance of 300 mm. Plates were developed using standard techniques.

2.4.2. Microdensitometry.

The microdensitometer used in this study was an ASBA image analyser (Fig. 2.10.). The Leitz Image Analyser consists of a microscope (Leitz

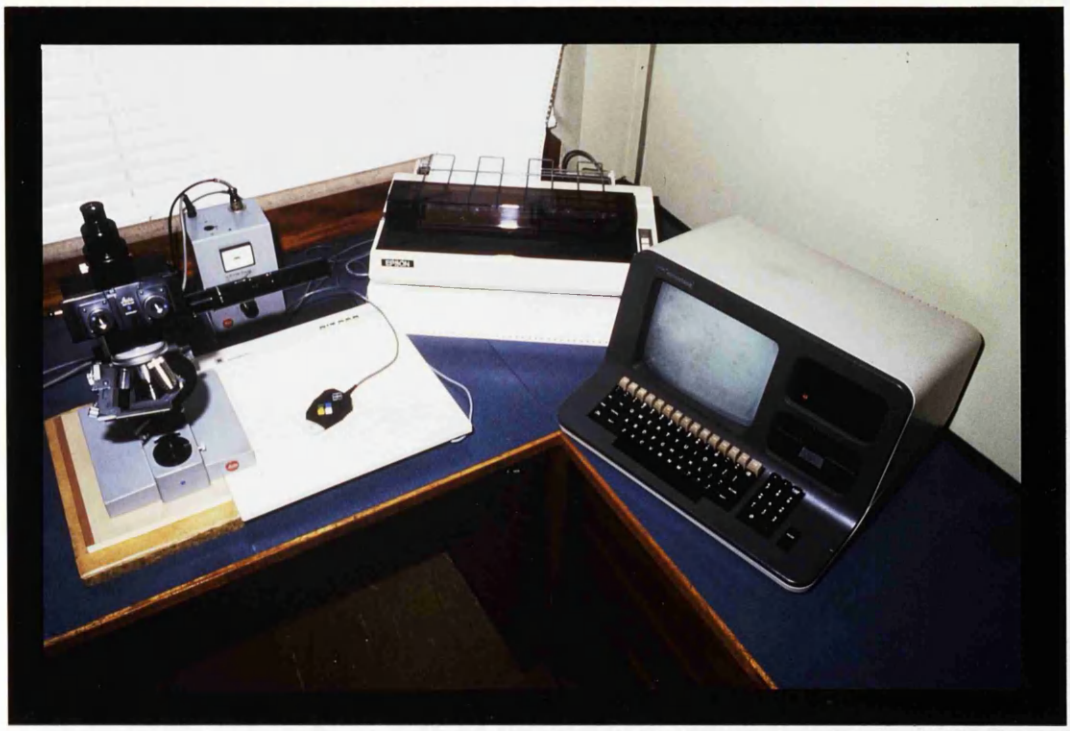


Fig. 2.8. Computerised planimetry equipment used for measurement of surface area of enamel slab.

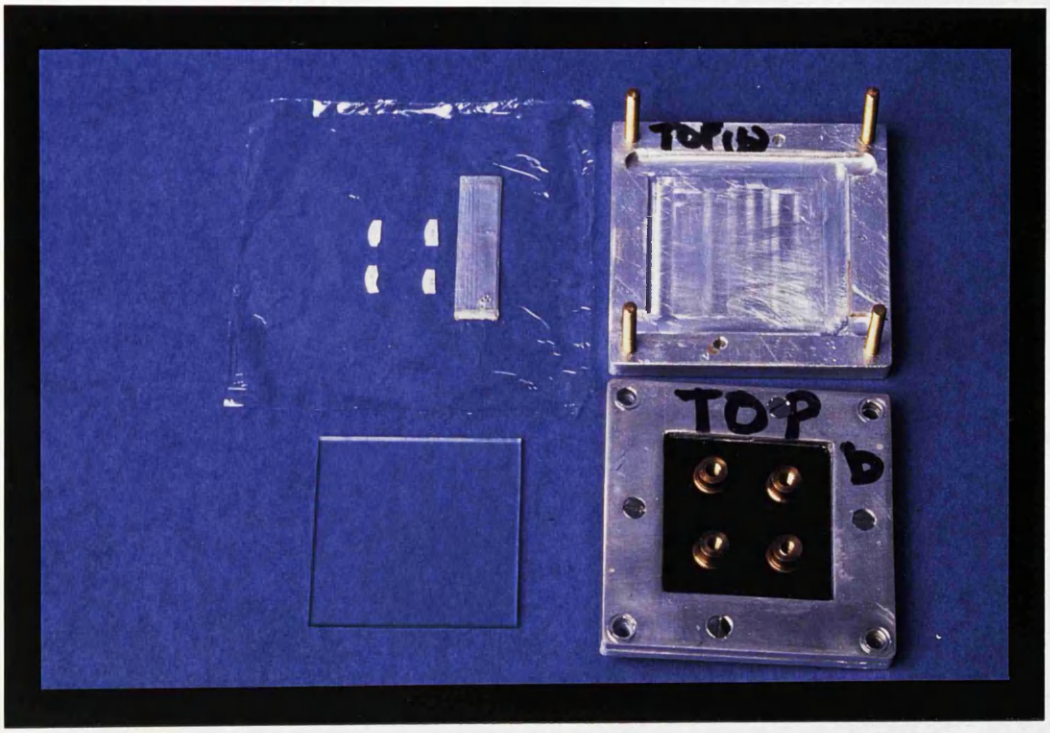


Fig. 2.9. Enamel sections and aluminium step-wedge with radiographic plate and plate holder used in microradiography of sections.

Fig. 2.10. **Image analyser used for microdensitometric analysis of microradiographs.**

Dialux 22) with a stabilised power supply, and a video camera (ASACA Corporation Type 700BE) mounted on the microscope. The analyser unit, controlled by a Z8002 microprocessor, allowed the video signals from the camera to be digitised into 256 grey levels with a resolution of 256 x 256 pixels. The image was then transferred to a BBC-B micro-computer for analysis and storage of results.

The microdensitometric methods described by Strang and co-workers (1987) were used to analyse the radiographs. The radiographic plate was placed on the microscope stage with the enamel site under study in the field of view. The intensity of the light source was first increased to saturate the video camera and the passage of light to the camera was then excluded by the interposition of an opaque card. Thus the two extremes of light intensity were entered into the microprocessor and this enabled the unit to be set up so that the 256 grey level range covered the region of interest.

The plate was then moved so that the thickest aluminium wedge (ie, the 300 μm portion of the step wedge) was in the microscopic field and an area in the centre of the screen, 64 x 64 pixels (192 x 192 μm) was sampled, and the average grey level for the wedge calculated and transferred to the BBC computer. Grey level values were then obtained for each of the other wedges in a similar manner.

The enamel site was then repositioned in the field of view, and the video camera rotated so that the surface of the enamel was as parallel to the top of the screen as possible. The image was then digitised into 256 x 256 pixels which were transferred to the BBC computer. Here the grey level values of the lesion appeared in a colour coded form. The width of the area to be examined could then be adjusted by positioning vertical lines on the screen on either side of the site of interest (Fig. 2.11.). The computer then calculated the average microdensitometric profile within these lines and displayed a profile on the right hand side of the screen.

Horizontal lines were then positioned over the image to limit the profile information stored in the computer. At this stage the profile was still in terms of grey values. A printout of the image on the screen was then taken so that the area of enamel examined at the baseline could be accurately re-located for analysis on the three-week radiograph. The

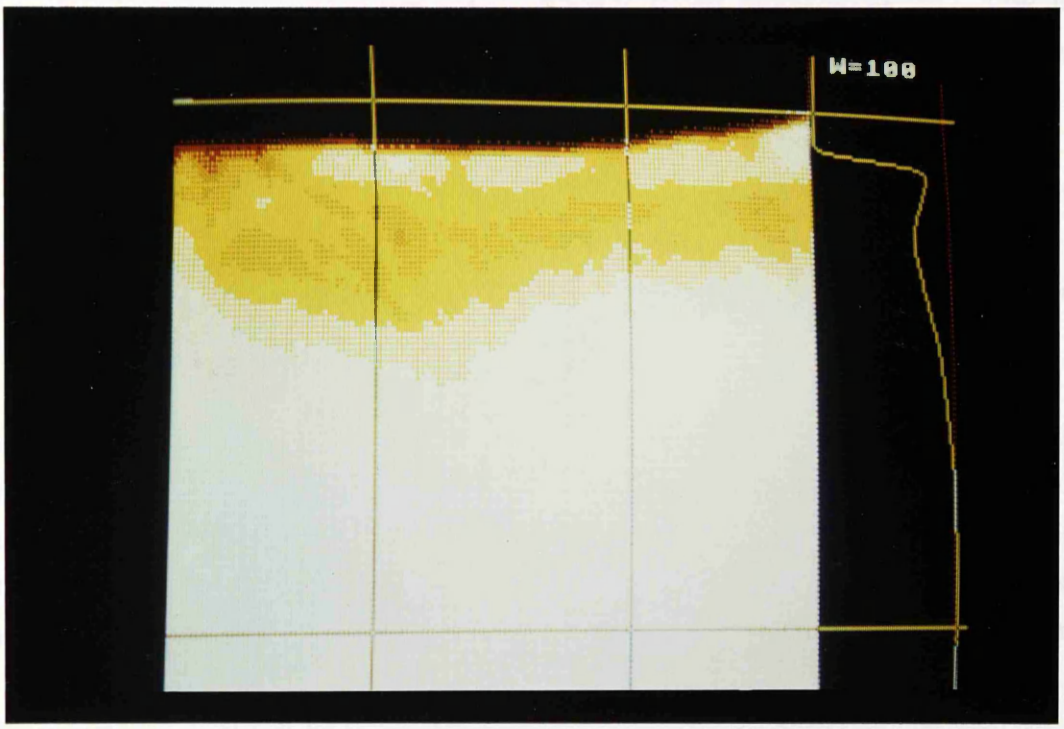


Fig. 2.11. **Image of enamel lesion shown on computer monitor with vertical lines positioned to determine area of analysis.**

grey levels of the enamel profile were converted to percent volume mineral by using the equation of Angmar and co-workers (1963), (see Appendix I).

Printouts of the enamel profiles at baseline and after the three-week experimental period were made for each site, and the mineral content at each time assessed. The parameters measured were the surface zone (SZ) and the lesion body (LB), in units of percent volume mineral, and the total mineral loss (Δz) in units of % vol min x μm (Fig. 2.12.). The limits of the total mineral loss were taken from the 20 % level of the initial slope of the profile to a point (S) in the sound enamel.

Quantification of the amount of mineral loss which had occurred during the experimental period was made by subtracting the baseline values for each parameter from the final values. The values quoted in the results reflect the net mineral loss, and are expressed as SZ, LB and Δz.

Mineral loss data was presented in two forms, based on the total mineral loss (Δz) and the change in mineral profile. The results of the former were divided into four groups, with Δz Group 1 containing sites experiencing demineralisation of less than 200 Δz units; Group 2 containing sites of 200 - 499 units; Group 3, 500 - 999 units and Group 4 comprising those sites where demineralisation exceeded 1,000 % vol min x μm.

The lesion profiles were also distributed among four groups. Where no change in profile was apparent, post-experimentally, the site was allocated to Group 1; sites showing slight surface softening of the enamel comprised Group 2; Group 3 contained sites where more extensive softening of the surface occurred and sites where subsurface demineralisation had occurred were allocated to Group 4. An example of each lesion profile group is shown in Fig 2.13.

2.5. Microbiological Procedures.

2.5.1. Plaque Sampling from Enamel Sections.

At the end of an experimental period, the appliance was removed from the

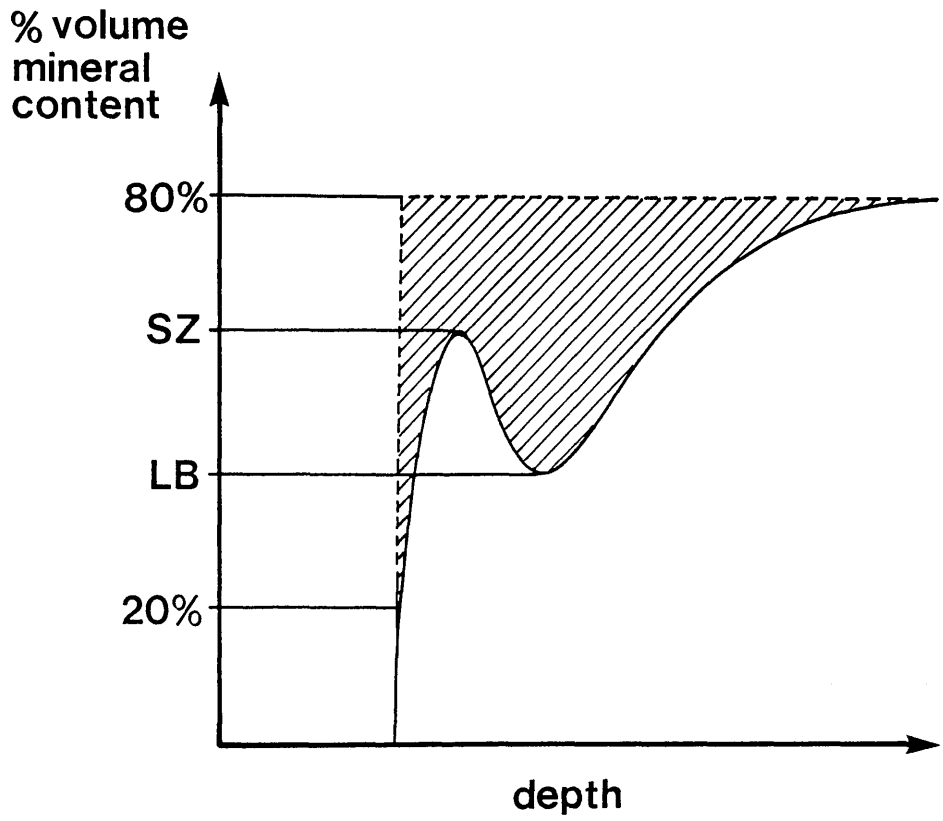
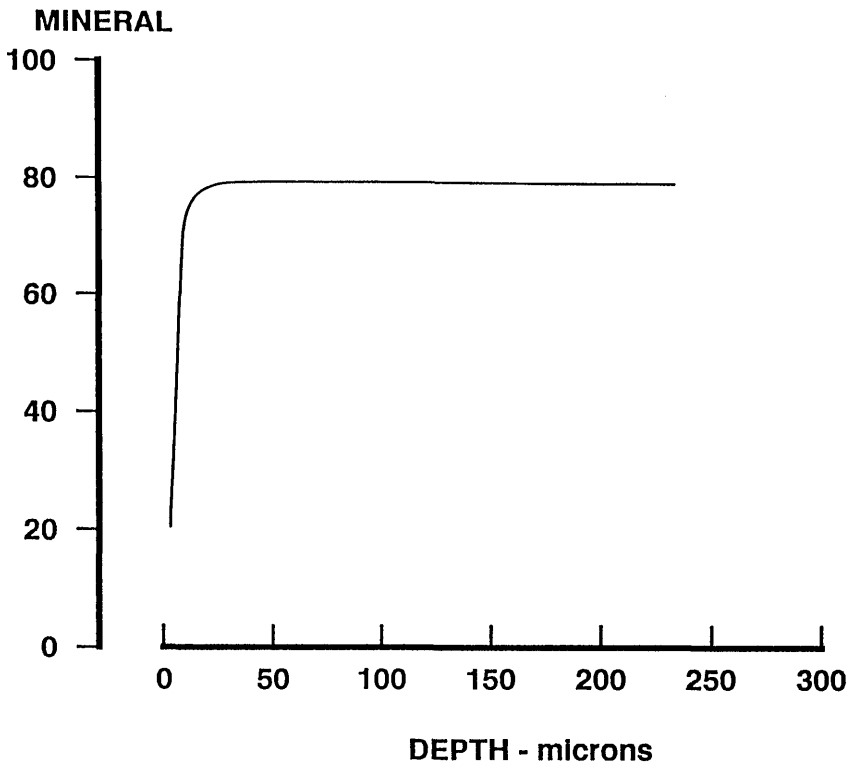


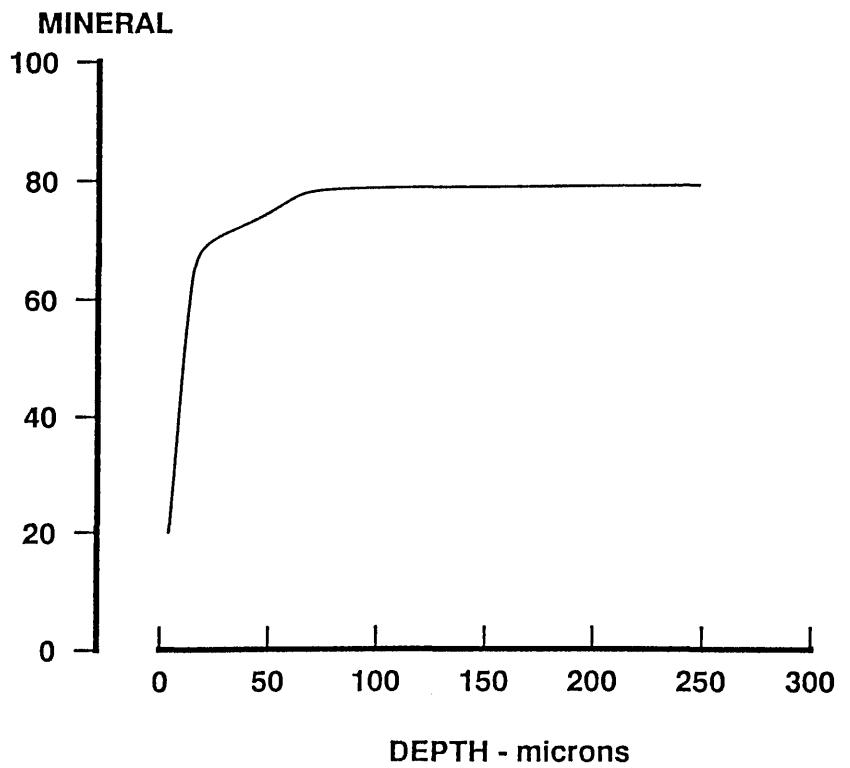
Fig. 2.12. **Microdensitometric profile of enamel showing parameters used to measure enamel demineralisation. i] SZ - Surface Zone (% vol min), ii] LB - Lesion Body (% vol min), iii] Δz - Total mineral loss (the shaded area).**

Fig. 2.13. Examples of lesion profiles obtained from in situ study, a] Group 1 - no apparent change, b] Group 2 - slight surface softening, c] Group 3 - extensive surface softening, d] Group 4 - subsurface demineralisation.

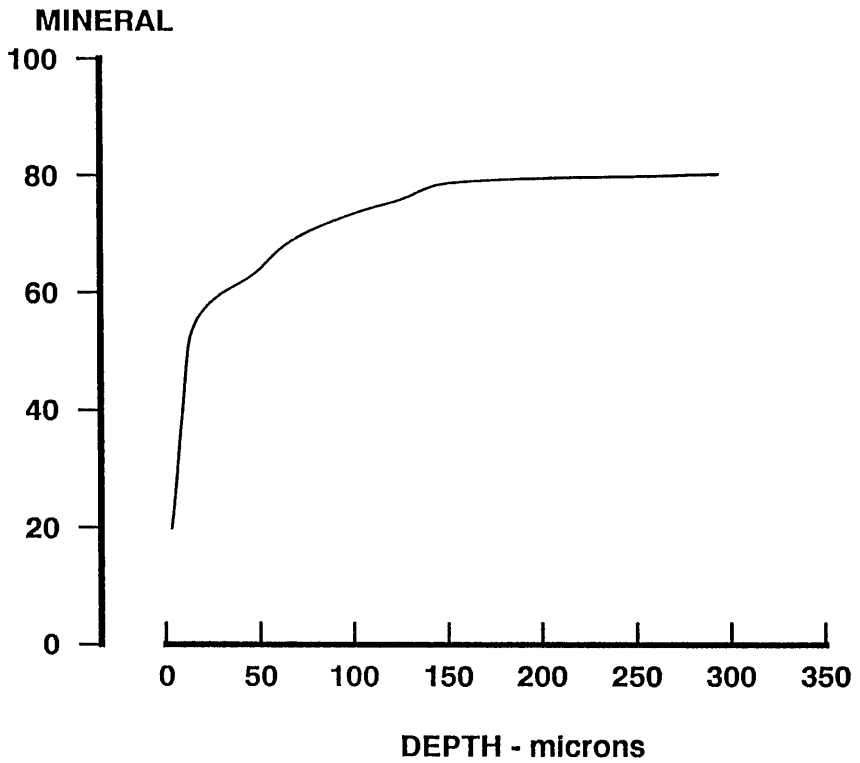
a]



b]



c]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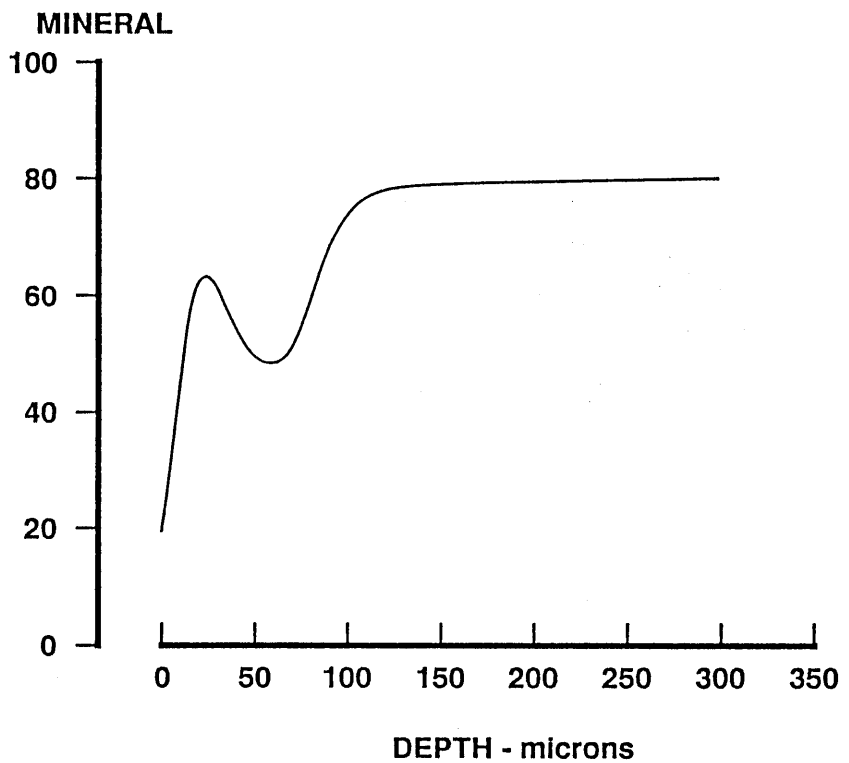
DEPTH - microns

d]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mouth and saliva gently washed from the trough area using a pasteur pipette containing 5 ml of physiological saline. The excess saline was removed by carefully applying sterile cotton wool pellets to the edge of the trough area. The diagram of the layout of the tooth sections prepared at the start of the experiment (see Section 2.3.6.) was then consulted and plaque overlying each enamel window was removed accurately using a sterile excavator (No. 243, Ash, England) and placed immediately in a labelled plastic bijou bottle containing 1 ml of Anaerobic Blood Broth (ABB) (Gibco-Europe, Paisley, Scotland), (see Appendix II).

2.5.2. Plaque Sampling from Enamel Slabs.

After removing superficial saliva from the trough area and referring to the initial diagram as described in 2.5.1., the plaque which had accumulated on the surface of each enamel slab was removed using a sterile dental excavator (No. 243, Ash, England). The plaque was then placed in 1 ml ABB. After sampling was completed, a proportion of the slabs were removed from the appliance and the enamel face pressed on to the surface of a blood agar plate in order to determine what percentage of the flora was retained on the enamel surface.

2.5.3. Plaque Dispersion and Dilution.

Each plaque sample was dispersed immediately in the ABB by sonication for 15 s at a setting of 1.5 (Ultrasonic Sonicator, Heat Systems Ultrasonics, Plainview, N.Y.) followed by vortex mixing for 30 s (Fisons Scientific Apparatus, Leicestershire, England). Thereafter, 10-fold dilutions from neat to 10^{-6} were carried out in ABB using an Eppendorf micropipette (BDH Laboratory Suppliers, Glasgow, Scotland) with 15 s vortex mixing between each dilution.

2.5.4. Plate Inoculation and Culturing.

From each sample, 50 µl of each dilution from 10^{-3} to 10^{-6} were inoculated on to 7.5 % Trypticase Soy Blood Agar (Gibco-Europe, Paisley, Scotland) supplemented with 1 % vitamin K and haemin (see Appendix II) using a spiral plater. Neat, 10^{-1} and 10^{-2} samples of plaque were

inoculated on to Mitis Salivarius Agar (Difco, Surrey, England) supplemented with 20 % sterile sucrose (BDH Chemicals Ltd., Poole, England) and 20 units bacitracin (Sigma Chemical Co. Ltd., Poole, England) per 100 ml, (MSB) (Appendix II), and on to Rogosa SL Agar (Difco, Surrey, England) (Appendix II).

The spiral plater (Model D, Don Whitley Scientific Ltd., West Yorkshire, England) expelled 50 µl of each dilution at a constant rate in a circular manner from the centre outwards (Fig 2.14.). Hence the concentration of the sample per surface area became progressively less on moving to the periphery.

The blood agar plates were incubated at 37° C for five days in an anaerobic cabinet (Don Whitley Scientific Ltd., West Yorkshire, England; Fig. 2.15.) in an atmosphere of 85 % N_2, 10 % H_2, 5 % CO_2. At this time the colonies present on a selected area of the plate were removed for subculture as described below and the plate incubated anaerobically for a further five days. Any colonies which appeared on the selected area during this time were also subcultured. The MSB and Rogosa plates were incubated at 37° C in an atmosphere of 5 % CO_2 in air for three days before counts were performed.

2.5.5. Identification of Isolates from Blood Agar Plates.

A blood agar plate with easily counted colonies was selected for each plaque sample (see Fig. 2.16.), and stamped with a grid (10 cm Spiral Systems Manual Counting Grid, Don Whitley Scientific Ltd., West Yorkshire, England) which divided the surface into zones representing known volumes of inoculum. An area containing 30 - 50 cfu was chosen, and each colony present in this area was subcultured on to a separate blood agar plate and incubated in the anaerobic cabinet until growth occurred. When a pure culture had been obtained, the following tests were carried out on each isolate;

1. Gram staining.

Colonies were removed from the plate using a sterile wire loop and emulsified in a loopful of sterile distilled water on a glass microscope

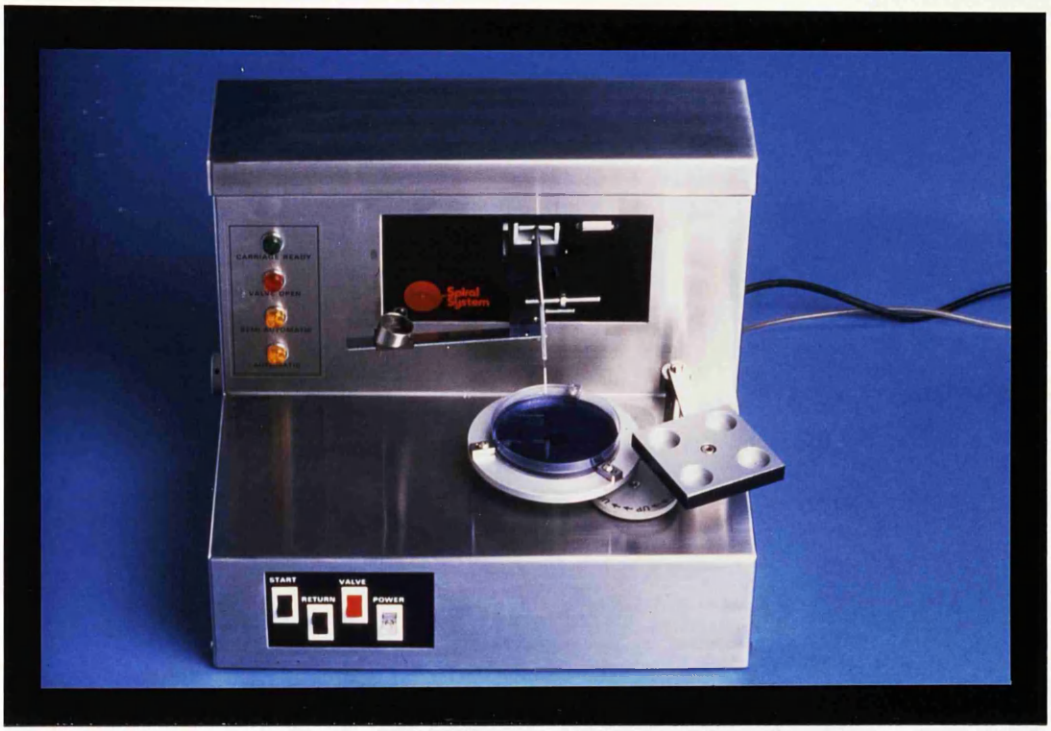


Fig. 2.14. Spiral plater in use with MSB plate in position.

Fig. 2.15. Anaerobic cabinet, used for incubation of blood agar plates.

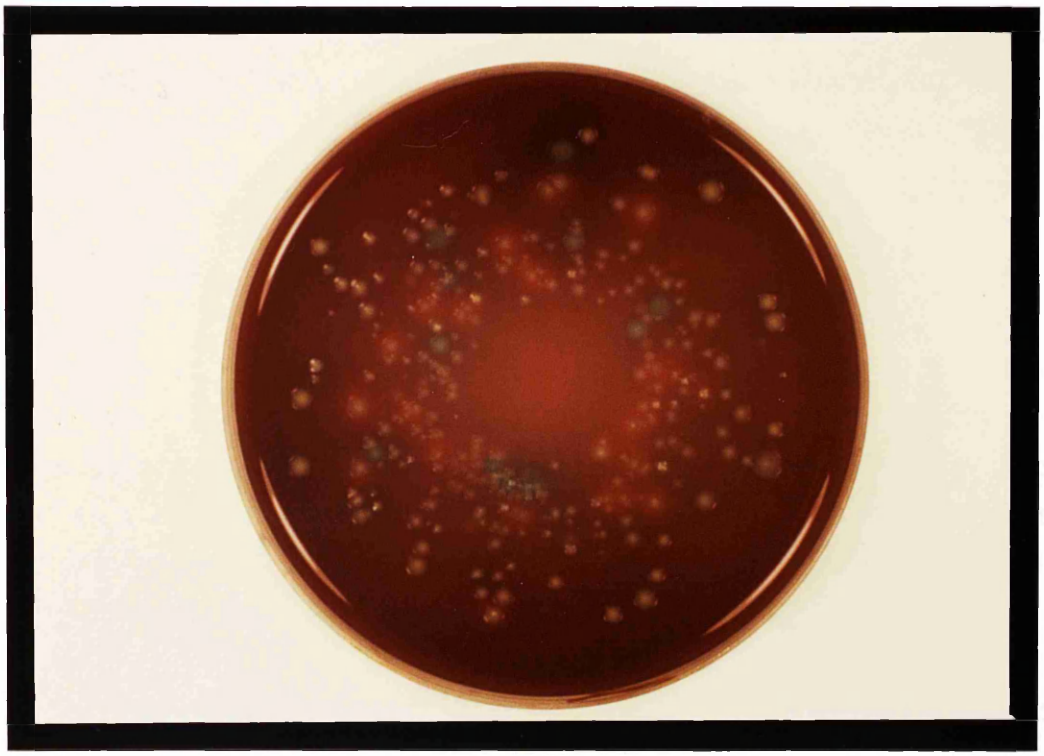


Fig. 2.16. **Inoculated blood agar plate following five days anaerobic incubation.**

slide. The smear was allowed to dry and was then heat-fixed by passing through a bunsen flame. The bacteria on the slide were then stained with a 0.5 % w/v solution of crystal violet. After one minute, the slide was rinsed with running tap water and then covered with Gram's iodine solution (Iodine 5 g, KI 10 g in 1 L distilled water) for a further minute. Decolourisation was then carried out under a stream of acetone (M & A Pharmachem Ltd., Bolton, England) and the smear then counterstained with a 5 % v/v solution of carbol fuschin for 2 - 3 min. The smear was then examined using an Olympus microscope (Tokyo, Japan) at a magnification of 1000 times and the morphology and staining characteristics noted. All the above chemicals, with the exception of acetone, were supplied by Clin-Tech Ltd, London, England.

2. Catalase production.

The test for catalase production was performed using a 3 % solution of hydrogen peroxide (BDH Chemicals Ltd., Poole, England). Colonies of the isolate were removed from the blood agar plate using a wire loop and added to a drop of the hydrogen peroxide solution on a microscope slide. If bubbles appeared within 10 s the test was considered positive, and if no visible reaction took place, the test was recorded as negative.

3. Atmospheric growth conditions.

Each isolate was incubated aerobically, in 5 % CO_2 in air and under anaerobic conditions in order to determine the atmospheric requirement necessary for growth.

From the results obtained from these procedures, appropriate biochemical tests were then performed for identification. The tests used for identifying the isolates are shown in diagrammatic form in Fig. 2.17.

1. API 20 Strep.

This system was used to identify catalase negative, facultatively anaerobic Gram positive cocci. The system combines 20 biochemical tests and is used to demonstrate enzymatic activity and the fermentation of sugars by the bacteria. The tests were carried out as follows:- a 24 hour blood

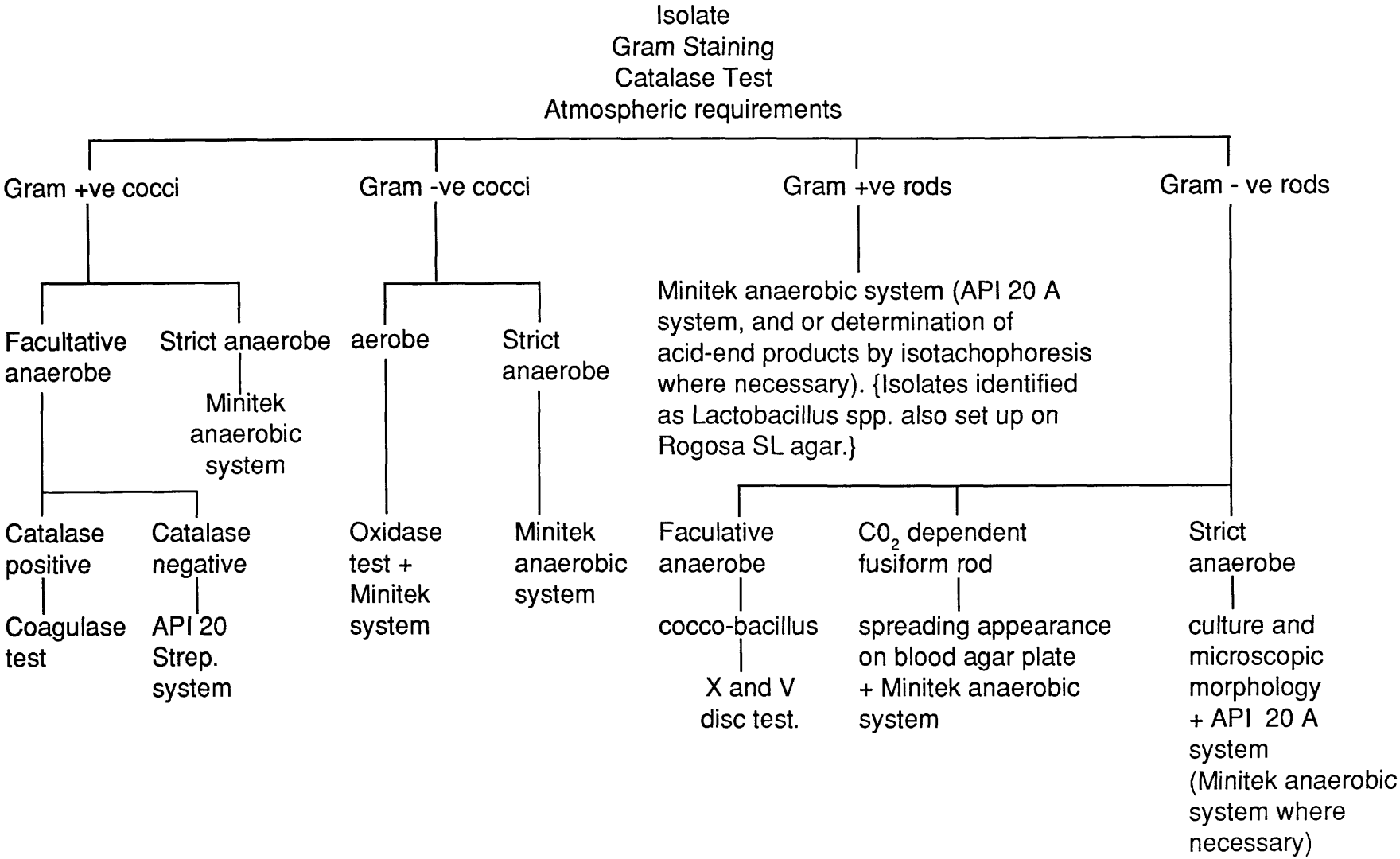


Fig. 2.17. Diagramatic representation of tests used for indentification of bacterial isolates

agar culture of the microorganism to be identified was grown at 37° C under anaerobic conditions. A sterile swab was used to harvest all the colonies from the plate, and a dense suspension of the isolate was made in 2 ml of sterile distilled water. Aliquots of the suspension were then distributed into the wells of the first half of the test strip as directed by the manufacturer. The remaining suspension was then transferred to an ampoule containing the API 20 Strep Medium and the new suspension was distributed into the remaining cupules. The cupules containing the substrates arginine to glycogen, were overlaid with mineral oil. The strip was then incubated at 37° C in 5 % CO_2 in air. The results were read at 4 and 24 hours, following the manufacturer's instructions (Fig. 2.18.), and the profile obtained (Fig. 2.19.) was entered into a computer (Apple Computer Inc., California, USA), programmed with the API identification disc. Thus the streptococci were identified to species level. If the computer regarded any profile as "doubtful", or as showing poor discrimination between species, the purity of the culture was checked, and the test repeated. If the same profile was obtained, the organism was reported at the genus level in a descriptive manner e.g. facultatively anaerobic streptococcus. Recent studies have suggested that some of the streptococcal species should be reclassified, and Schmidhuber and co-workers (1987) consider that species identified as Strep. sanguis II and Strep. mitis using the API 20 Strep System should be re-named Strep. oralis. This practice was adopted in the current study. A more detailed discussion on the taxonomy of the streptococcal species is to be found in Chapter III.

2. API 20 A.

The API 20 A system was used to identify the anaerobic Gram negative rods and to aid in the identification of Gram positive rods when results of tests using the Minitek system were equivocal. This system consists of cupules containing dehydrated substrates. Colonies of the test organism were removed from the blood agar plate and inoculated into the API 20A medium to produce a heavy suspension. Aliquots of this were then added to the tubes following the manufacturer's instructions, and the strip incubated in an anaerobic cabinet at 37° C for a minimum of 24 hours. Appropriate reagents were then added and the colour changes noted. A biochemical profile was obtained for the bacterium and was entered into

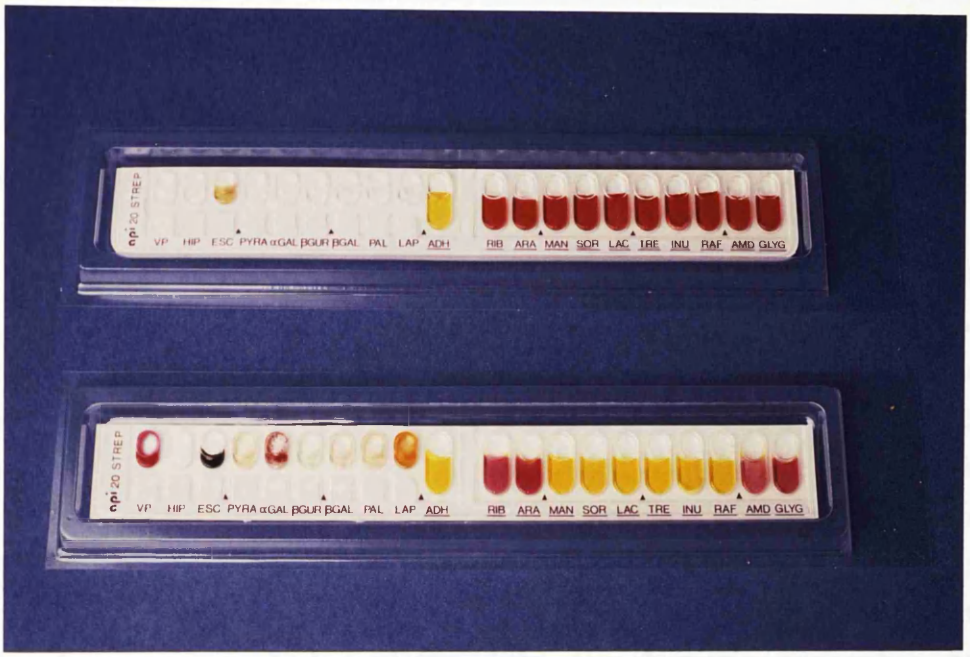


Fig. 2.18. **API strips following inoculation of bacterial suspension (above) and following 24 h incubation and application of reagents (below).**

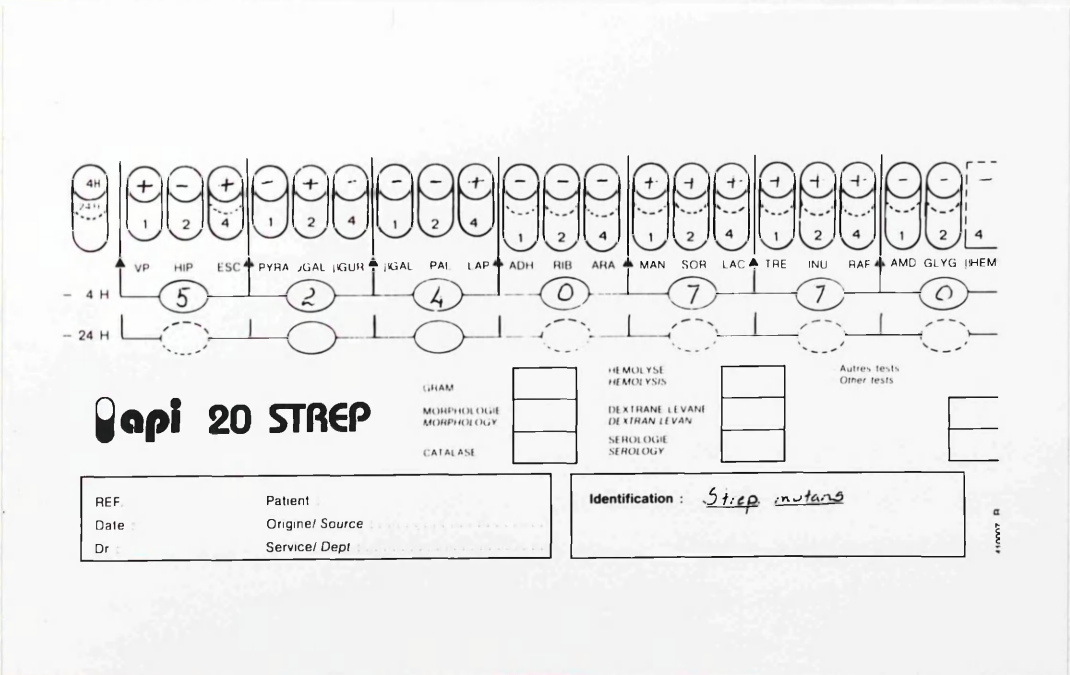


Fig. 2.19. **Biochemical profile obtained from Fig. 2.18.**

the Apple computer programmed with the API identification disc as described above. This, together with Gram staining characteristics and colonial and cultural morphology, was used for the identification of the organism.

3. Minitek anaerobic system.

The Minitek anaerobic system also utilises biochemical methods and was used for the differentiation of the strictly anaerobic Gram positive and negative cocci, the facultative and strictly anaerobic Gram positive rods, and on occasions, the Gram negative rods if the discrimination between genera was poor when using the API 20 A system. Following the manufacturer's instructions, discs impregnated with the test substrates were individually dispensed into wells of the Minitek plate. Colonies of the test bacterium were removed from a pure blood agar culture using a sterile dry swab and inoculated into the Minitek anaerobic broth to produce a dense suspension. A plastic pipette was then used to dispense a standard drop of this suspension into each well. After a minimum of 24 hours anaerobic incubation, biochemical reagents were added to the wells and the discs examined for specific colour changes, using the manufacturer's instructions (Fig. 2.20.).

The Minitek system contained the following biochemical tests:- fermentation of 15 carbohydrates, hydrolysis of aesculin, reduction of nitrate, and the production of indole and urease. The biochemical profile of the organism which was obtained was then compared with the manufacturer's differentiation tables to determine its identification.
When the identification of Gram positive rods had a confidence level of under 75 % using the Minitek system, and the purity of the culture had been checked, the isolate was inoculated into the API 20 A system (API Systems SA Montalieu-Vercieu, France). If discrimination at the genus level was still doubtful, the acid end products from glucose metabolism were determined by isotachophoresis (vide infra). If doubt remained at the species level, the organism was reported at the genus level.

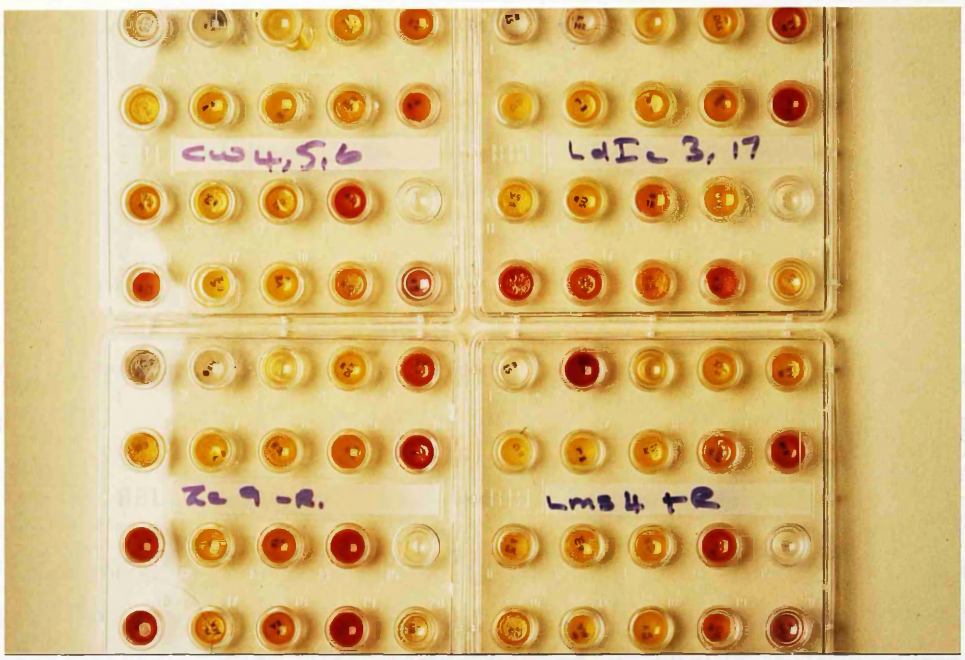


Fig. 2.20. **Impregnated discs in wells, used in Minitek anaerobic system, following 24 h anaerobic incubation and addition of reagents.**

Details of other laboratory procedures used to identify the isolates are :

1. Slide agglutination test.

This was carried out on the catalase positive, facultatively anaerobic Gram positive cocci, using the Roche Diagnostica Staph-Rapid Test (Roche Products Ltd, Welwyn Garden City, England). Colonies of the isolate were removed from a blood agar plate using a wire loop, and mixed with a drop of sensitised erythrocyte suspension and a drop of control erythrocyte suspension on a microscope slide. At the same time, a positive control using a type culture of Staphylococcus aureus, was also tested. The slides were gently rocked to and fro, and the test was considered positive if the sensitised erythrocyres clearly agglutinated within 15 s, whilst the control erythrocytes remained finely dispersed.

2. Oxidase disc test.

The identification of bacteria belonging to the Neisseriaceae family was based on microscopic appearance, catalase production, and on a positive oxidase disc test (A/S Rosco, Taastrup, Denmark). Colonies of the isolate were removed from the plate using a sterile dry swab and suspended in 3 ml of sterile distilled water in a plastic bijou. A disc was then added and the bijou shaken by hand for 5 s. The colour of the suspension and disc was noted, with the production of a purple colour within 30 s indicating a positive reaction. Also each isolate was set up in the Minitek system to determine carbohydrate utilisation, and was classified as being either saccharolytic or asaccharolytic within the Neisseria / Branhamella group.

3. X and V disc test.

This test was used for the differentiation of facultatively anaerobic Gram negative cocco-bacilli. Colonies were removed from the blood agar plate using a sterile swab and were suspended in sterile water which was then inoculated on to a nutrient agar plate (Gibco-Europe, Paisley, Scotland), deficient in both X and V Factors. Discs containing X, V, or both X and V (Mast Laboratories, Merseyside, England) were applied to the surface of the agar and the plate incubated in 5 % CO_2 at 37° C for a minimum of

24 hours. The growth pattern around the discs was used to differentiate Haemophilus species and Eikenella corrodens.

In some instances, following primary subculture of the isolate on to a blood agar plate, there was poor growth which was insufficient to allow appropriate biochemical tests to be set up for identification. On other occasions the discrimination between genera remained poor after carrying out a number of the above procedures. In such cases the isolate was reported as being "unidentified" within its staining and morphological group.

The organisms identified from each plaque sample obtained from an enamel section were expressed as a percentage of the total cfu. In the case of a sample from an enamel slab, in addition to the percentage of the total cfu, the isolates were also expressed as an absolute count per mm^2 of enamel surface.

2.5.6. Enumeration and Identification of Streptococcus mutans and Lactobacillus species.

On each MSB and Rogosa plate, the number of cfu with similar macroscopic colonial morphology was counted. This was carried out for each of the dilutions where colonies were present, and the mean calculated. Two representatives of each colonial type in each sample were identified using the API 20 Strep system for isolates from the MSB plates, and the Anaerobic Minitek System from the Rogosa plates. The number of Strep. mutans and Lactobacillus spp. present were calculated and expressed as a percentage of the total cultivable flora count which was obtained from the blood agar cultures for each sample.

2.6. Freeze-drying Procedure.

Representative colonies of each species isolated from each volunteer were freeze-dried and stored, employing the following methods, for use in subsequent studies. The code and identification of the isolate to be preserved was type-written on a card which was then cut into a narrow strip that fitted into a glass ampoule. A small wisp of cotton wool was placed loosely into the top of the tube, which was then sterilised using

dry heat at a temperature of 160°C for one hour. A 24 hour pure blood agar culture of the isolate was removed using a sterile dry swab and inoculated into 10 ml ABB supplemented with 10 % horse serum. Using a pasteur pipette, 0.5 ml of this inoculum was then placed in a pre-labelled sterile glass ampoule. The culture was subsequently freeze-dried using an Edwards EF4 Modulyo freeze-dryer (Edwards High Vacuum, Crawley, England). Freezing was carried out, both during centrifugation and when the vials had stopped spinning, until a pressure of 1.3×10^{-1} mbar or below was achieved. The vials were then constricted using an Edwards Ampoule Constrictor and loaded on to the secondary drying head on the freeze-dryer. Drying then took place under a vacuum for one hour and each ampoule was then sealed using an Edwards Flamemaster hand torch, and stored at room temperature.

When the freeze-dried cells were required, the appropriate ampoule was scored just above the cotton-wool bung using a diamond pencil and then broken open along this line. The cotton-wool was removed and the deposit reconstituted in 2 ml of ABB. A sterile plastic pipette was used to remove 50 µl aliquots of this suspension which were inoculated on to blood agar plates and incubated anaerobically at 37°C until growth occurred. Before a culture was used in an experiment, its purity and identity was confirmed by the appropriate biochemical tests as described in Section 2.5.5.

2.7. Plaque Acid Anion Profiles and pH Measurements.

2.7.1. Acid Anion Estimations.

Volunteers, both from Glasgow Dental School and Unilever Research Port Sunlight, were instructed to consume neither food nor drink from the evening before, until after plaque sampling for acid anion profiles had been carried out the following morning. At that time, the appliance was removed from the mouth and fasting plaque samples taken from the enamel slabs using a sterile dental excavator (No. 243, Ash, England). On some occasions, plaque was also collected from the natural dentition. Each sample was immediately placed within the top of a plastic vial containing moist cotton-wool in its base. The lid was then closed and the sample stored at -20°C until analysis was performed.

The appliance was re-inserted and the volunteer then rinsed the mouth with 10 ml of a 10 % w/v sucrose solution for one minute. After 6 min the appliance was removed and further plaque samples taken and stored as above at -20°C to reduce the metabolism of substrates by the plaque bacteria.

The samples collected from Glasgow subjects were processed within 4 hours of harvesting, whilst those obtained in Port Sunlight were placed in a flask containing crushed ice and transported to Glasgow within 6 hours of collection. There they were transferred to a -20° C freezer and processed within the subsequent 24 hour period.

Each plaque sample was removed from its plastic container and placed in a pre-weighed plastic vial which was then reweighed and the weight of sample calculated. The plaque was then mixed with 10 µl of the leading electrolyte (vide infra) and centrifuged at 20,000 g for 15 min in a refrigerated MSE High Speed 18 centrifuge (MSE Ltd., Crawley, Sussex, England). The supernatant fluid was then removed and stored at -20°C.

Plaque acid anions were analysed by isotachophoresis using a LKB 2127 Tachophor (LKB Instruments, Bromma, Sweden, Fig. 2.21.) fitted with conductivity and ultraviolet detection systems (Geddes and Weetman, 1981). The separation took place in a 610 mm Teflon capillary tube of 0.5 mm diameter maintained at a constant temperature of 12°C.

The leading electrolyte was 5 mM hydrochloric acid adjusted to pH 4.2 by the addition of 6-amino-n-hexanoic acid. Hydroxypropylmethylcellulose (0.2 % w/v) was added to sharpen the boundaries between acids. The terminating electrolyte was 4 mM n-octanoic acid adjusted to pH 5.5 by the addition of 2-amino-2 (hydroxymethyl) propane-1, 3-diol (Tris). These chemicals were supplied by BDH Chemicals Ltd., Poole, England, with the exception of the hydroxypropylmethylcellulose which was supplied by the Sigma Chemical Co. Ltd., Poole, England.

Standard solutions of 5 mM formic, pyruvic, phosphoric, lactic, succinic, acetic and propionic acids, supplied by BDH Chemicals Ltd., Poole, England, were run through the system and calibration curves obtained from which

Fig. 2.21. **Tachophor and chart recorder used for analysis of plaque acid anions.**

the zone lengths of the tracings could be converted into nanomolar concentrations (Fig. 2.22.).

Samples of plaque supernatant were then run through the tachophor at 50 μA. The traces obtained were then analysed, and the amount of each acid expressed in nmol/mg wet weight of plaque. When the weight of collected plaque was under 0.8 mg, accurate determination of the concentrations of the acids could not be achieved, therefore when this occurred, only the lactate / acetate ratio of the sample was calculated.

2.7.2. Plaque pH Measurements.

Overnight fasting plaque samples were removed from the enamel slabs mounted on the in situ appliance and the plaque pH measured using the one-drop electrode technique described by Geddes & MacFadyen (1981), a modification of that described by Frostell (1969). The electrode (Beckman Instruments, Glenrothes, Scotland) and Corning 120 pH meter (Corning Ltd., Halstead, Essex, England) were first calibrated using buffer solutions of pH 4 and pH 7 (BDH Chemicals Ltd., Poole, England) and a calibration curve plotted for pH against mV. Small plaque samples of approximately 1 mg were mixed with 10 μl of deionised water in the glass electrode. The reference electrode was then inserted into the plaque and the mV reading recorded after one minute. This reading was then converted to a pH measurement using an equation derived from the calibration curve. Plaque samples were taken at baseline and at 1, 3, 6, 10, 15, 20 and 30 minutes following a 10 % sucrose mouth rinse, and from these a Stephan curve was drawn by plotting pH against time.

2.8. Statistical Analyses.

The intra-oral appliance was employed for studies on the effect of treatment conditions and time on microbial plaque composition, and the relationship between enamel demineralisation and proportions of bacteria in the overlying plaque, utilised a model in which other factors were also involved, which may have influenced the bacterial composition. An additive main effects model was therefore used to analyse the data obtained. This system was used to model variables such as i] side of appliance, ii] position within acrylic trough area, iii] experimental run,

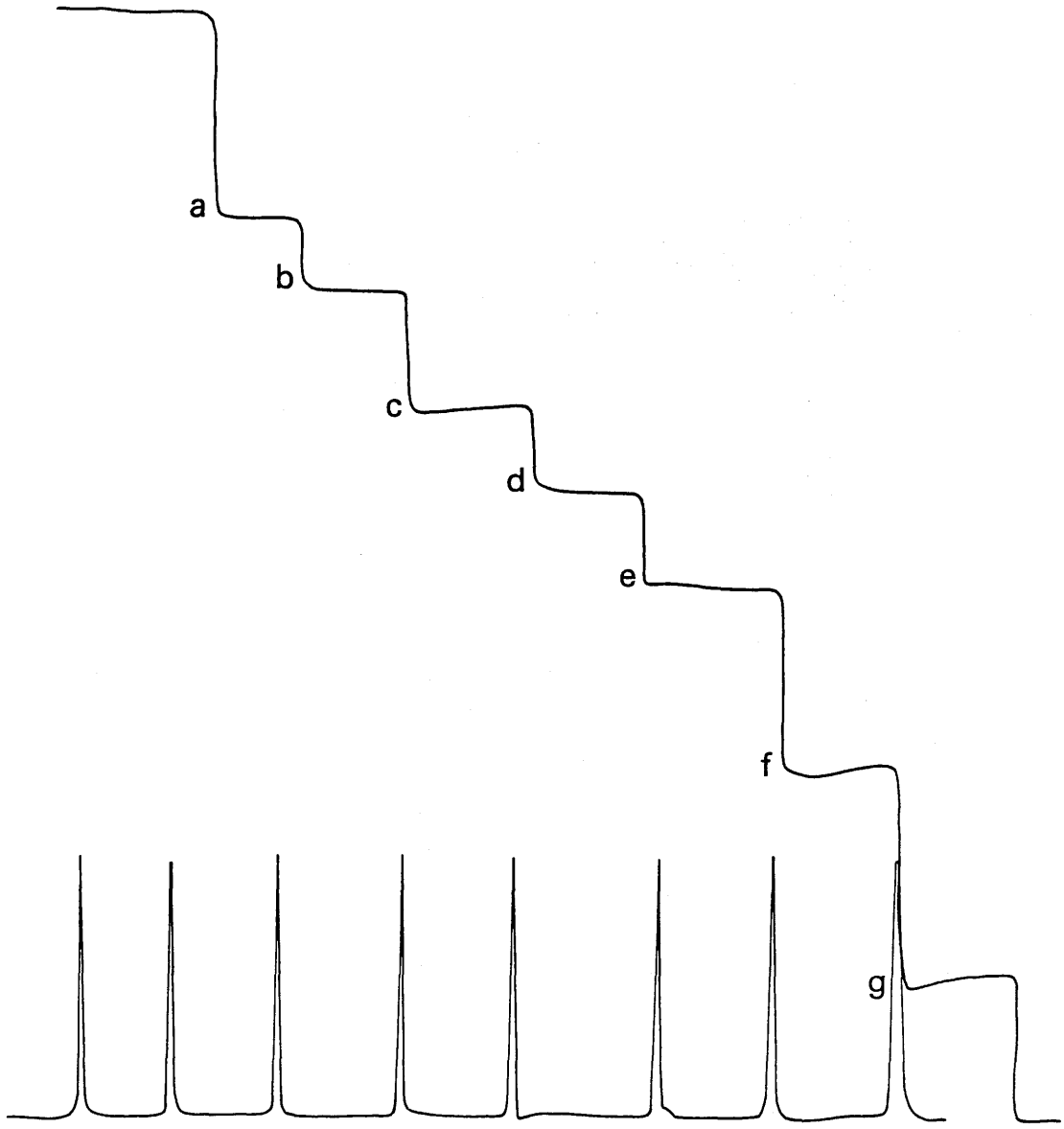


Fig. 2.22. **Example of tracing, used in calibration, obtained from standard acid solutions, a] formate, b] pyruvate, c] phosphate, d] lactate, e] succinate, f] acetate, g] propionate.**

and iv] subject, in addition to the factors under study. For each of these variables an F-test was performed, taking into account all other factors which may have had an effect at that particular time. The statistical package used was the Generalised Linear Interactive Modelling System (GLIM) (Numerical Algorithms Group Ltd., 1987).

Where an effect of one of the factors under study was evident, eg with regard to treatment conditions on the levels of a particular organism, the differences among the treatment effects were investigated by a pairwise multiple comparison procedure, using the estimates of the treatment effects and their standard errors, in the presence of all other factors.

In general, throughout this thesis, the effect of each factor on plaque microflora has been tabulated for each study, and where a significant effect was found, the level of significance has been shown. When this occurred with regard to one of the factors under study, eg time or treatment conditions, pairwise multiple comparison procedures were carried out as described above, and those variables within each factor which caused a significantly different effect from that of the other variables, were detailed in the text of the results, rather than in tabular form.

The absolute counts of bacteria per mm^2 enamel slab surface were log transformed prior to analysis. To enable transformation of zero values to be carried out, it was necessary to add a value to the raw data for each organism before log transforming. The minimum detection level for each organism under study in each plaque sample was calculated with reference to the dilution factor of the plate used for the identification of the bacteria, the volume of inoculum used to inoculate the area of the plate from which the colonies were removed for identification and the surface area of the enamel slab from which the plaque sample had been removed. Various proportions of the minimum detection level for each organism were then added to the raw data, and probability plots of the log transformed data were constructed to determine which additive component to the raw data gave adequate assumption of normality to the transformed data. From the results of this procedure, it was decided to add half of the minimum detection level to the raw absolute counts of each organism per mm^2 enamel surface, before log transforming.

CHAPTER III

PRELIMINARY STUDIES.

3.1. Introduction.

This chapter describes the experiments that were performed during the development of the appliance model, and gives details of investigations carried out to test the validity of some of the methods which were to be used in the main in situ studies.

3.2. Total Counts and Identification of Plaque Microflora - A Reproducibility Experiment.

3.2.1. Introduction.

A variety of different techniques has been used by research workers to collect, transport, disperse, culture and identify the flora of dental plaque samples, and these differences may account in part for the wide variation in the microbial composition of plaque which has been reported in different studies (reviewed by Johnson and Murphy, 1983).

The main study was designed to standardise sampling from discrete sites without contamination from adjacent areas, and to permit processing of samples to take place immediately after plaque collection. However, some of the other techniques which were to be used in the enumeration and identification of microorganisms, such as dispersal, dilution, culture conditions and the selection of areas of the agar plate for the identification of colonies, are known to affect the reported composition of a plaque sample.

This reproducibility study was therefore undertaken :

1] to examine the effect of plaque dispersion by sonication on
   a) the retrieval of individual species from a plaque sample and
   b) the total counts of bacteria isolated, and

2] to determine the composition of the microflora obtained
   a) from different dilutions of the same plaque sample and

b) from different areas on the same blood agar plate, to ascertain how representative of the original sample, was an area of a blood agar plate containing 30-50 colonies.

3.2.2. Methods.

An enamel section, mounted on an intra-oral appliance, was inoculated with Strep. mutans, previously isolated from the subject's own plaque, during the first 28 hours of a one week experimental period, as will be described later in Section 4.3.2.

After one week, a plaque sample was removed from the enamel section (see Section 2.5.1). The protocol followed in processing the sample is shown in diagrammatic form in Fig. 3.1. After removal, the plaque was placed immediately in 1 ml of ABB and vortex-mixed for 30 s. Two separate 0.1 ml aliquots were removed and, from each of these, dilutions in ABB from 10^{-1} to 10^{-6} were carried out, with a further 15 s vortex mixing between each dilution. The remaining 0.8 ml was then sonicated for 15 s at a setting of 1.5, and two separate 0.1 ml aliquots were then immediately removed and dilutions carried out as above. These procedures resulted in two dilution runs for both vortex-mixed only and vortex-mixed and sonicated samples. Each of the 10^{-1} to 10^{-6} dilutions for each of the four sets of dilutions was then spiral plated on to two blood agar plates. These were incubated anaerobically at 37°C (see Section 2.5.4.) and were then examined. From each set of blood agar plates, two plates of different dilution factor, each containing a minimum of 60 colonies, were chosen. This gave a total of four plates for each of the four sets of dilutions - eight plates from the votex-mixed and sonicated sample and eight from the original sample which had received only a vortex mixing. On each plate two sites were selected, each site containing between 30 and 50 colonies, in an area representing a known volume of inoculum. This gave a total of 32 sites. The total cfu's present in the original sample was then calculated separately from each of these sites, and the isolates identified and expressed as a percentage of the total cultivable flora in each site.

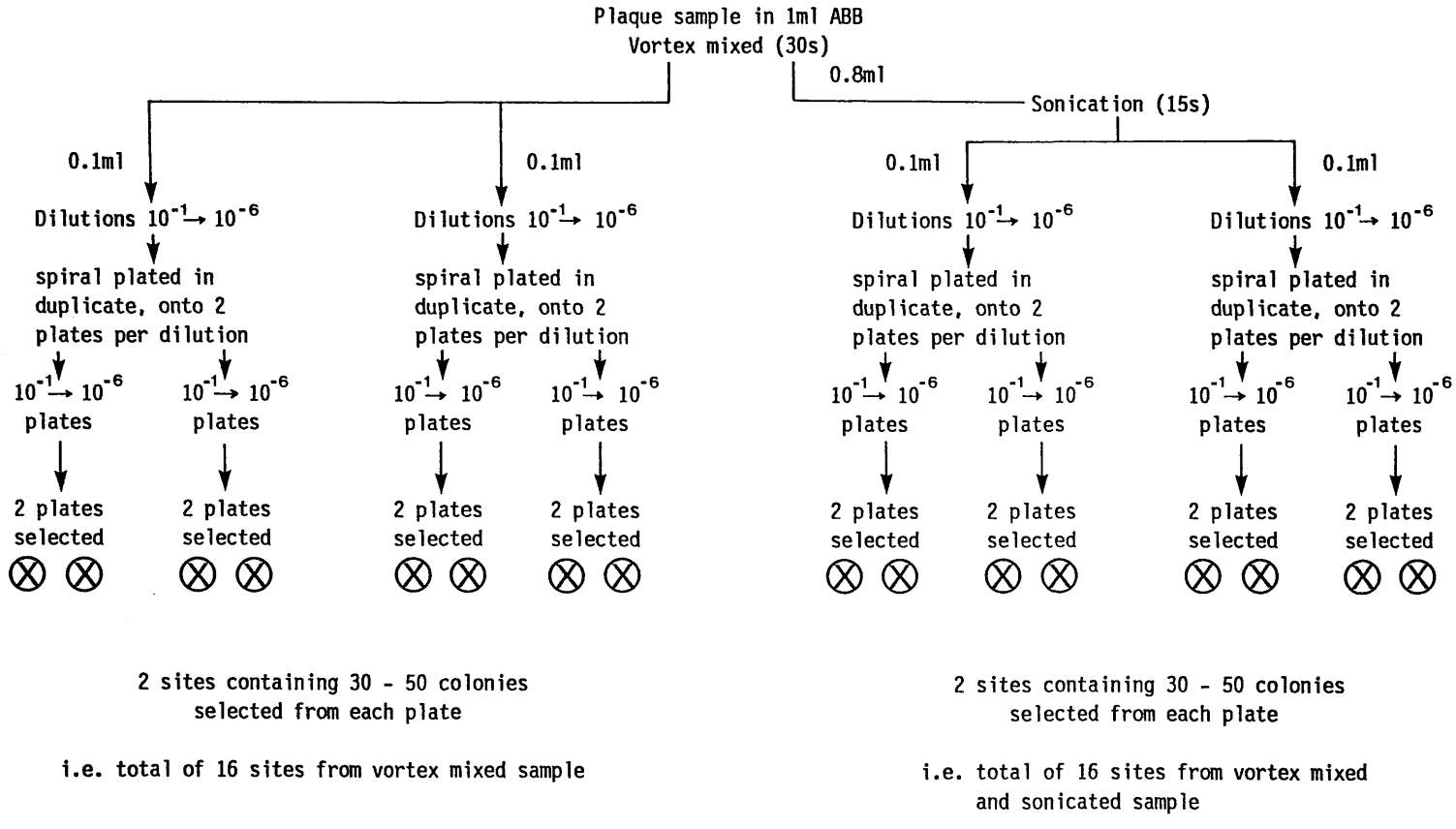


Fig. 3.1. Diagramatic representation of experimental protocol followed in reproducibility study.

3.2.3. Results.

Bacteria Isolated.

The predominant bacteria isolated from the 32 samples are shown in Tables 3.1. to 3.5. The column headings used in these Tables are detailed in Table 3.1. A Propionibacterium species was isolated from one of the vortex and sonicated samples where it accounted for 4.8 % of the total cfu, but otherwise the isolates shown in the Tables comprised the total of the anaerobic cultivable flora obtained from the samples. Strep. mutans was isolated from all 32 samples, and the mean proportion was 17.0 % for the vortex mixed samples, and 22.4 % for the vortex mixed plus sonicated samples.

Effect of Method of Plaque Dispersal.

The isolation frequency, mean, standard deviation and range of the proportions of the bacteria isolated from the 16 vortex-mixed and 16 vortex-mixed and sonicated samples are shown in Table 3.1. The predominant group of organisms, following both dispersal methods, was positive cocci, which accounted for 83.7 % of organisms in the vortex-mixed samples, and 88.1 % in the vortex-mixed and sonicated samples. Negative cocci and positive bacilli comprised only a very small proportion of the total cultivable flora, while the Gram negative bacilli formed the second largest group after both dispersal methods. The absolute counts of the organisms, expressed as $\log_{10}$ cfu are also shown. No significant differences were found between the proportions of the genera and species isolated from the samples. Although the negative bacilli proportion was slightly lower in the sonicated sample (not statistically significant, see Table 3.6.), the actual number of negative bacilli in the sonicated sample was higher. The percentage of the positive cocci and the total counts were significantly higher ($p < 0.05$ and $p < 0.001$ respectively) in the samples which had been dispersed by sonication in addition to vortex mixing. The statistical analysis of Tables 3.1. to 3.5. are shown in Table 3.6.

The standard deviations of the mean percentage counts were slightly lower for each of positive and negative coccal and bacillary groups in the sonicated samples compared to the vortex-mixed only samples.

The increase in the mean total counts of organisms following sonication of the sample was by a factor of five, with the Gram positive cocci counts being 5.4 times higher. The counts of Gram negative cocci increased by the smallest amount (1.7 times), whilst the change in both Gram positive and negative bacillary counts was almost four-fold.

Effect of Dilution Run.

For both vortex-mixed and vortex-mixed samples with sonication, 2 aliquots were removed for dilution and analysis. The results obtained from these parallel runs are shown in Tables 3.2. and 3.3. The total counts obtained from the second dilution run of the vortex-mixed samples were significantly higher ($p < 0.05$), but otherwise no significant differences were found between the proportions of the organisms following either dispersal method, or the total counts in the sonicated samples (see Table 3.6.).

Effect of Dilution Factor.

A comparison was made between the organisms isolated using different dilution factors. In the vortex-mixed sample, 10^{-3} and 10^{-4} dilutions were used, while in the sonicated samples 10^{-4} and 10^{-5} dilutions were used, because of the higher counts of organisms isolated. The results are given in Tables 3.4. and 3.5. and show no significant differences in proportion or counts of organisms between the two concentrations for either dispersal method.

Effect of Duplicate Plating and Plate Position.

Each dilution for both dispersal methods was plated on to two separate plates. The isolates present in the pairs of plates for the chosen dilutions, outlined above, were compared, and no significant differences found in either proportions or counts.

Isolates were removed from two positions on opposite sides of each of these plates, and again no significant differences were found.

VORTEX

	F[a]	Mean[b] (SD)[c]	Range	Log_{10} cfu[e]
+ve cocci	16/16	83.7 (5.6)	74.5 - 94.7	7.00
-ve cocci	2/16	0.6 (1.6)	ND[d]- 5.0	4.86
+ve bacilli	6/16	2.2 (3.6)	ND - 11.8	5.42
-ve bacilli	16/16	13.5 (5.5)	5.3 - 25.4	6.21
S. mutans	16/16	17.0 (9.0)	5.9 - 40.0	6.31
S. sanguis	16/16	53.5 (14.2)	30.0 - 76.5	6.81
S. oralis	14/16	14.0 (9.6)	ND - 26.9	6.23
Veillonella	2/16	0.6 (1.6)	ND - 5.0	4.86
Actinomyces	6/16	2.2 (3.6)	ND - 11.8	5.42
Bacteroides	12/16	7.2 (6.3)	ND - 18.2	5.94
Capnocytophaga	11/16	4.3 (4.0)	ND - 12.9	5.71
Total (log_{10} cfu)		7.08 (0.3)	6.50 - 7.86	

VORTEX + SONICATION

	F	Mean (SD)	Range	Log_{10} cfu
+ve cocci	16/16	88.1 (4.0)	79.4 - 94.4	7.73
-ve cocci	2/16	0.2 (0.6)	ND - 1.9	5.09
+ve bacilli	7/16	1.7 (2.1)	ND - 5.0	6.02
-ve bacilli	16/16	10.0 (4.4)	4.2 - 19.0	6.79
S. mutans	16/16	22.4 (8.7)	7.9 - 40.0	7.14
S. sanguis	16/16	55.1 (16.6)	19.0 - 80.0	7.53
S. oralis	12/16	10.6 (14.5)	ND - 47.6	6.82
Veillonella	2/16	0.2 (0.6)	ND - 1.9	5.09
Actinomyces	5/16	1.1 (1.7)	ND - 4.5	5.83
Bacteroides	14/16	7.1 (5.2)	ND - 17.5	6.64
Capnocytophaga	10/16	2.6 (3.5)	ND - 13.6	6.20
Total (log_{10} cfu)		7.79 (0.2)	7.52 - 8.15	

[a] - Isolation frequency ; [b] - Mean percentage of total cfu ;
[c] - Standard deviation ; [d] - Not detected ; [e] - Mean log_{10} cfu.

Table 3.1. Effect of method of plaque dispersal on predominant cultivable microflora, n=16.

VORTEX DILUTION 1

	F	Mean (SD)	Range	Log_{10} cfu
+ve cocci	8/8	82.8 (4.6)	76.5 - 90.0	6.82
-ve cocci	2/8	1.2 (2.1)	ND - 5.0	4.98
+ve bacilli	4/8	3.8 (4.6)	ND - 11.8	5.48
-ve bacilli	8/8	12.3 (4.3)	5.9 - 17.6	5.99
S. mutans	8/8	19.2 (11.9)	5.9 - 40.0	6.18
S. sanguis	8/8	50.4 (16.1)	30.0 - 76.5	6.60
S. oralis	7/8	13.4 (8.6)	ND - 26.1	6.03
Veillonella	2/8	1.2 (2.1)	ND - 5.0	4.98
Actinomyces	4/8	3.8 (4.6)	ND - 11.8	5.48
Bacteroides	6/8	4.9 (4.1)	ND - 13.0	5.59
Capnocytophaga	6/8	5.8 (4.7)	ND - 12.9	5.66
Total (log_{10} cfu)		6.90 (0.2)	6.50 - 7.15	

VORTEX DILUTION 2

	F	Mean (SD)	Range	Log_{10} cfu
+ve cocci	8/8	84.7 (6.6)	74.5 - 94.7	7.19
-ve cocci	0/8	ND	ND	ND
+ve bacilli	2/8	0.6 (1.0)	ND - 2.3	5.04
-ve bacilli	8/8	14.7 (6.6)	5.3 - 25.4	6.43
S. mutans	8/8	14.8 (4.4)	7.7 - 23.1	6.43
S. sanguis	8/8	55.2 (13.1)	34.6 - 70.8	7.00
S. oralis	7/8	14.7 (11.1)	ND - 26.9	6.43
Veillonella	0/8	ND	ND	ND
Actinomyces	2/8	0.6 (1.0)	ND - 2.3	5.04
Bacteroides	6/8	9.5 (7.6)	ND - 18.2	6.24
Capnocytophaga	5/8	3.5 (3.2)	ND - 7.7	5.80
Total (log_{10} cfu)		7.26 (0.3)	6.90 - 7.86	

Table 3.2. Effect of dilution run for vortex mixed samples on predominant cultivable microflora, n=8.

VORTEX + SONICATION
DILUTION 1

	F	Mean (SD)	Range	Log_{10} cfu
+ve cocci	8/8	87.5 (3.9)	82.6 - 94.4	7.72
-ve cocci	0/8	ND	ND	ND
+ve bacilli	4/8	2.0 (2.2)	ND - 4.8	6.08
-ve bacilli	8/8	10.5 (3.8)	5.6 - 17.4	6.80
S. mutans	8/8	18.6 (4.1)	10.0 - 24.0	7.05
S. sanguis	8/8	57.1 (22.6)	19.0 - 80.0	7.54
S. oralis	4/8	11.7 (19.4)	ND - 47.6	6.85
Veillonella	0/8	ND	ND	ND
Actinomyces	3/8	1.4 (2.0)	ND - 4.5	5.93
Bacteroides	6/8	6.9 (5.8)	ND - 17.4	6.62
Capnocytophaga	4/8	3.0 (4.7)	ND - 13.6	6.26
Total (log_{10} cfu)		7.78 (0.2)	7.57 - 7.94	

VORTEX + SONICATION
DILUTION 2

	F	Mean (SD)	Range	Log_{10} cfu
+ve cocci	8/8	88.7 (4.4)	79.4 - 92.5	7.75
-ve cocci	2/8	0.4 (0.8)	ND - 1.9	5.40
+ve bacilli	3/8	1.4 (2.1)	ND - 5.0	5.95
-ve bacilli	8/8	9.6 (5.2)	4.2 - 19.0	6.78
S. mutans	8/8	26.1 (10.6)	7.9 - 40.0	7.22
S. sanguis	8/8	53.1 (8.6)	41.8 - 66.7	7.53
S. oralis	8/8	9.4 (8.4)	2.4 - 28.4	6.77
Veillonella	2/8	0.4 (0.8)	ND - 1.9	5.40
Actinomyces	2/8	0.7 (1.5)	ND - 4.2	5.64
Bacteroides	8/8	7.3 (4.9)	3.8 - 17.5	6.66
Capnocytophaga	6/8	2.2 (2.0)	ND - 5.9	6.14
Total (log_{10} cfu)		7.80 (0.2)	7.52 - 8.15	

Table 3.3. Effect of dilution run for vortex mixed and sonicated samples on predominant cultivable microflora, n=8.

VORTEX 10^{-3}

	F	Mean (SD)	Range	Log_{10} cfu
+ve cocci	8/8	85.2 (6.3)	76.5 - 94.7	7.03
-ve cocci	0/8	ND	ND	ND
+ve bacilli	4/8	3.0 (4.2)	ND - 11.8	5.58
-ve bacilli	8/8	11.8 (5.3)	5.3 - 18.5	6.17
S. mutans	8/8	16.5 (10.9)	5.9 - 40.0	6.32
S. sanguis	8/8	53.4 (15.4)	30.0 - 76.5	6.83
S. oralis	6/8	15.4 (10.6)	ND - 26.3	6.29
Veillonella	0/8	ND	ND	ND
Actinomyces	4/8	3.0 (4.2)	ND - 11.8	5.58
Bacteroides	5/8	6.9 (6.9)	ND - 16.3	5.94
Capnocytophaga	6/8	5.1 (4.2)	ND - 12.9	5.81
Total (log_{10} cfu)		7.10 (0.4)	6.50 - 7.86	

VORTEX 10^{-4}

	F	Mean (SD)	Range	Log_{10} cfu
+ve cocci	8/8	82.3 (4.7)	74.5 - 88.9	6.96
-ve cocci	2/8	1.2 (2.1)	ND - 5.0	5.13
+ve bacilli	2/8	1.3 (2.9)	ND - 8.3	5.16
-ve bacilli	8/8	15.2 (5.5)	8.3 - 25.4	6.23
S. mutans	8/8	17.5 (7.3)	5.9 - 27.8	6.29
S. sanguis	8/8	52.3 (14.3)	34.6 - 70.8	6.77
S. oralis	8/8	12.7 (8.9)	2.1 - 26.9	6.15
Veillonella	2/8	1.2 (2.1)	ND - 5.0	5.13
Actinomyces	2/8	1.3 (2.9)	ND - 8.3	5.16
Bacteroides	7/8	7.5 (6.1)	ND - 11.8	5.92
Capnocytophaga	5/8	4.2 (4.2)	ND - 11.8	5.67
Total (log_{10} cfu)		7.05 (0.2)	6.83 - 7.34	

Table 3.4. Predominant cultivable plaque microflora, isolated from 10^{-3} and 10^{-4} dilutions of vortex mixed samples, n=8.

VORTEX + SONICATION 10^{-4}

	F	Mean (SD)	Range	Log_{10} cfu
+ve cocci	8/8	87.5 (5.3)	79.4 - 94.4	7.58
-ve cocci	2/8	0.4 (0.8)	ND - 1.9	5.24
+ve bacilli	3/8	1.0 (1.6)	ND - 4.0	5.64
-ve bacilli	8/8	11.1 (5.4)	5.6 - 19.0	6.68
S. mutans	8/8	23.0 (8.9)	7.9 - 35.8	7.00
S. sanguis	8/8	53.8 (16.2)	24.0 - 70.4	7.37
S. oralis	6/8	10.6 (14.4)	ND - 38.0	6.65
Veillonella	2/8	0.4 (0.8)	ND - 1.9	5.24
Bacteroides	8/8	9.2 (5.8)	3.7 - 17.5	6.60
Capnocytophaga	6/8	1.9 (1.5)	ND - 4.0	5.92
Actinomyces	3/8	1.0 (1.6)	ND - 4.0	5.64
Total (log_{10} cfu)		7.64 (0.1)	7.52 - 7.78	

VORTEX + SONICATION 10^{-5}

	F	Mean (SD)	Range	Log_{10} cfu
+ve cocci	8/8	88.8 (2.4)	85.7 - 91.7	7.89
-ve cocci	0/8	ND	ND	ND
+ve bacilli	4/8	2.3 (2.5)	ND - 5.0	6.30
-ve bacilli	8/8	8.9 (3.2)	4.2 - 13.6	6.89
S. mutans	8/8	21.8 (9.1)	10.0 - 40.0	7.28
S. sanguis	8/8	56.4 (18.1)	19.0 - 80.0	7.69
S. oralis	6/8	10.5 (15.6)	ND - 47.6	6.96
Veillonella	0/8	ND	ND	ND
Actinomyces	2/8	1.1 (2.0)	ND - 4.5	5.98
Bacteroides	6/8	5.0 (3.7)	ND - 10.0	6.64
Capnocytophaga	4/8	3.4 (4.8)	ND - 13.6	6.47
Total (log_{10} cfu)		7.94 (0.1)	7.83 - 8.15	

Table 3.5. Predominant cultivable plaque microflora, isolated from 10^{-4} and 10^{-5} dilutions of vortex mixed and sonicated samples, n=8.

	Dispersal	Dilution Run	Dilution Factor	Duplicate Dilution	Plate Position
+ve cocci	*	NS	NS	NS	NS
-ve cocci	NS	NS	NS	NS	NS
+ve bacilli	NS	NS	NS	NS	NS
-ve bacilli	NS	NS	NS	NS	NS
S. mutans	NS	NS	NS	NS	NS
S. sanguis	NS	NS	NS	NS	NS
S. oralis	NS	NS	NS	NS	NS
Veillonella	NS	NS	NS	NS	NS
Actinomyces	NS	NS	NS	NS	NS
Bacteroides	NS	NS	NS	NS	NS
Capnocytophaga	NS	NS	NS	NS	NS
Total	***	*	NS	NS	NS

NS = Not significant ; * = $p < 0.05$; *** = $p < 0.001$.

Table 3.6. Statistical analysis of effects of factors on isolation of plaque microorganisms in repeatability experiment.

3.2.4. Discussion.

The complexity and variability of dental plaque referred to in Chapter I, together with the many practical problems inherent in quantitative studies on mixed microbial populations, make it difficult to obtain accurate and reproducible data (reviewed by Hardie and Bowden, 1976). Cultivation techniques are unable to isolate all the microorganisms present in a particular plaque sample, and there is a wide range in the reported proportion of organisms, expressed as a percentage of the total microscopic count, which can be cultured from plaque samples, with some studies finding as little as 10 - 20 % (reviewed by Manganiello et al., 1977). Improvements in anaerobic culture conditions have increased the proportion of recoverable organisms in recent years.

However, many factors play a role in accounting for the variation in the reported plaque composition obtained from a particular site. These factors include i] the sampling technique employed; ii] the method used to transport the sample to the laboratory; iii] the technique used to disperse the plaque; iv] the culture methods employed including the culture medium used and the atmospheric growth conditions, v] the number of colonies selected and the method of their selection, and vi] the identification techniques.

There are two basic sampling methods for obtaining plaque. In one, relatively large pooled samples are obtained from many sites in the mouth, whilst in the other, smaller specimens are obtained from discrete sites. The former method has the advantage of providing sufficient material to allow accurate weighing and the use of many different tests such as biochemical analysis and microbial counts (reviewed by Johnson & Murphy, 1983). However, pooling of samples may obscure important site variations, and does not permit the plaque flora to be related to the condition of discrete areas of underlying tooth enamel. A variety of different techniques and materials have been employed for obtaining samples. These are dependent to some extent on the accessibility of the site to sampling. Some of the instruments used to obtain supragingival plaque include dental floss (Shklair et al., 1974), abrasive strips (Bowden et al., 1975), dental probes (van Houte & Duchin, 1975), wooden toothpicks

(Mikkelsen & Poulsen, 1976) scalers (Mikkelsen et al., 1981), and pieces of wire (van Palenstein Helderman, 1981).

The appliance study described in Chapter IV, was designed to allow accurate plaque sampling from discrete "windows" on the enamel sections, so the microflora could be related to changes in the mineralisation of the underlying enamel. A small dental excavator was used to remove plaque overlying these sites, and in the main study, two enamel sections were mounted on each side of the appliance with a space of 1-2 mm between them. This permitted plaque to be related to discrete enamel sites, and also allowed easier instrumentation, thus reducing the contamination from adjacent areas. The plaque overlying the enamel slabs was also removed using a dental excavator, and in this case the whole surface of the enamel was scraped with the instrument, thus allowing the microbial data obtained to be expressed as a count per mm^2 enamel surface as well as by percentage of the total anaerobic cultivable flora. Immediately prior to plaque sampling, some workers flush the surface of the plaque with physiological saline, in an attempt to remove loosely bound bacterial salivary contaminants (Socransky et al., 1977), and Nyvad & Kilian (1987) consider that this rinsing procedure is necessary in studies on early plaque colonisation. This was therefore carried out in the appliance study on every occasion prior to sampling, as described in Section 2.5.1.

For optimal isolation of viable organisms, plaque samples should be processed in the laboratory as soon as possible after removal from the mouth (Hardie & Bowden, 1976). In some studies, particularly epidemiological ones, delays are inevitable and it is therefore necessary to place the plaque samples in a suitable medium which will maintain the viability of the organisms without allowing selective overgrowth of some species. Several such transport media have been used, including Reduced Transport Fluid (Syed & Loesche, 1972; Loesche et al., 1975) and simple nutrient broths containing cysteine (Nyvad & Kilian, 1987). In all the experimental studies carried out and described in this thesis, the subjects removed their intra-oral appliance in the laboratory. Processing of the plaque therefore took place immediately after sampling, and all inoculated plates were in their appropriate incubator after a maximum of 30 min following initial plaque sampling from the enamel sites. The specimens of plaque were placed in Anaerobic Blood Broth (Appendix II)

immediately after sampling and all subsequent dilutions were carried out in this medium.

To carry out quantitative studies on the composition of dental plaque, disaggregation of the bacteria should be attempted in order to produce a suspension containing single microbial cells prior to making dilutions and plating out. However, the complex nature of plaque with its heterogeneous combinations of microbial sizes and shapes, together with the varying susceptibility of the organisms to physical forces and exposure to oxygen, means it is difficult to find a suitable method which will adequately disperse plaque without selectively favouring the survival and growth of specific groups of organisms. Many techniques have been employed and include vortex mixing, shaking with glass beads, tissue grinding and homogenisation, the use of hypodermic syringes and needles and sonic oscillation (reviewed by Hardie & Bowden, 1976; Manganiello et al, 1977). In addition to physical means, attempts have been made to separate the organisms by chemical or enzymatic means, eg, by the addition of EDTA to the dispersing fluid.

Tissue grinders and sonic oscillation appear to be the most commonly used physical methods, with the former producing less force, and therefore perhaps preserving the viability of higher numbers of fragile organisms, but at the same time proving less efficient than the latter at dispersing microbial cells.

The organisms considered most at risk from sonication procedures are the Gram negative bacteria, and it is thought that the cell wall composition and the relative inability of these organisms to adhere to one another and form clumps, influences their susceptibility to the sonication forces (Olsen & Socransky, 1981). The harmful effect of sonic oscillation is therefore thought to be particularly important in plaque studies associated with periodontal disease, since Gram negative rods and filaments form a larger proportion of the flora than in supragingival plaque, and are believed to be the major pathogens.

In the present study, the effect on microbial proportions and counts of dispersing a one week old plaque sample by vortex mixing and 15 seconds of sonication, compared to vortex mixing alone, was examined. As one of

the protocols of the main appliance demineralisation study described in Chapter IV involved the inoculation of Strep. mutans on to the enamel sites to be sampled, and as this is one of the organisms implicated in the aetiology of caries, it was particularly important to ensure that this organism was present so that reproducibility of its identification could be examined. Consequently, Strep. mutans was inoculated on to the test site for this experiment, and this will therefore have influenced the relative proportions of the various organisms isolated.

The results showed that the proportion of Gram positive cocci was significantly higher when sonication was applied after vortex mixing, and this is in agreement with findings from other studies (reviewed by Hardie & Bowden, 1976), while there was no difference in the mean percentage of negative bacilli between the two groups. However, the dispersal procedures were carried out in aerobic conditions, as facilities for performing this under anaerobic conditions were not available, and this may therefore have reduced the proportion of viable oxygen-sensitive Gram negative bacteria in both dispersal groups. In addition, the samples collected throughout the study were supragingival, and therefore the organisms reported as being most fragile, such as Treponema, Selenomonas and Wolinella species (Olsen & Socransky, 1981) were less likely to be present, and since the investigation concerned the cariogenic potential of plaque, rather than its potential to cause periodontal disease, selective culture of these organisms was not performed.

The standard deviations of the mean proportions of the Gram positive and negative cocci and bacilli were all slightly lower in the sonicated group, suggesting that a slightly more even distribution of the microbial cells may have been achieved with this method of plaque dispersal.

The greatest increase in the mean counts of organisms present in the vortex-mixed plus sonicated samples compared to the vortex-mixed only samples occurred with the Gram positive cocci, where a five-fold increase was seen. This was presumably due to the dispersion of clumps and chains of streptococcal species, while at the same time preserving the viability of individual cells. Gram negative genera such as Veillonella species are far more sensitive to the lethal effects of sonication than streptococci (Robrish et al., 1976) and this finding was borne out in the

present study as their increase in viable count following sonic oscillation was only by a factor of 1.7. The four-fold increase in Gram negative bacilli obtained following 15 s sonication was of approximately the same magnitude as for the positive bacilli. Although the former group are considered more sensitive than the latter, and a smaller increase was anticipated, the two Gram negative genera present in the samples, namely Bacteroides and Capnocytophaga species, have been found to be relatively resistant to sonic energy (Olsen & Socransky, 1981).

The mean total viable counts of organisms isolated from the duplicate dilution runs following sonication showed very little variation, whilst a significant difference between the total $\log_{10}$ cfu counts was obtained from the duplicate runs following vortex mixing alone. This again suggests that a more even dispersal of particles was achieved by sonic oscillation.

A comparison of the proportion and counts of organisms obtained from plates inoculated with different dilution factors was carried out to determine whether the concentration of bacterial cells inoculated on to a culture plate affected the growth of the organisms, as it was felt that the bacteria from an inoculum with lower concentration may have more nutrients available than ones present on a more densely populated plate. However, no significant difference was found in either the relative proportions of the different groups of organisms or in the total viable counts obtained from the samples.

This short study, which involved the identification of organisms from 32 different sites on blood agar plates, each containing a minimum of 30 colonies, was carried out on one original plaque sample, and showed that some variation in the reported composition of the flora from these repeat measurements took place. This finding was inevitable, due in part to the very small fraction of bacteria actually identified from the original sample and the many factors outlined previously. However, although some variation did occur, within each dispersal group there was no significant difference in the relative proportion of the identified organisms between the different dilution runs, dilution factors or positions on the culture plate from which the colonies were subcultured.

Therefore, although this study was carried out on only one occasion due to the very large number of identifications involved (approximately 1,300 isolates identified), and despite the limitations outlined above, it was considered acceptable, in subsequent studies, to attempt to determine the bacterial composition of plaque by identifying all the colonies from a single region, containing 30 - 50 cfu, on a single blood agar plate.

The aim of the main appliance study was to investigate the relationship of the composition of the plaque flora to enamel demineralisation, in which Gram positive cocci and bacilli are considered the most important pathogens. Although it is recognised that some organisms are more resistant than others to sonic oscillation, borne out to some extent in this preliminary study with an increase in proportion of positive cocci, the proportion of Gram negative cocci and bacilli were not significantly reduced. Therefore, in view of this, and the fact that this method of plaque dispersal had appeared to distribute the particles more evenly in the broth suspension prior to dilution and plating, it was decided that sonication of the samples, as described in Section 2.5.3., would be carried out in all subsequent studies.

Culturing of Organisms.

Since the microorganisms found in plaque have different growth requirements and are present in widely differing proportions, the culture media and atmospheric conditions employed in any study will depend on the purpose of the investigation and the organisms under investigation (Hardie & Bowden, 1976). In the main study, which concerns the association of plaque flora and early demineralisation, the objectives were to identify the organisms most numerous in the plaque as well as the specific bacteria which have been implicated in the aetiology of caries. Therefore, a blood-based non-selective medium (described in Chapter II) was used to determine the total cultivable flora and predominant isolates, while selective media were employed for the culture of Strep. mutans and Lactobacillus spp. which were likely to be present in low numbers, and therefore might be missed on densely populated non-selective plates. The non-selective plates were incubated in an anaerobic chamber, which has been shown to produce the highest viable counts of organisms from dental plaque (Hardie & Bowden, 1976). The selective plates were cultured under

aerobic conditions with the addition of 5 % CO_2, one of the commonly used methods employed in the isolation of Strep. mutans and lactobacilli. Although this culture method for non-selective media may fail to isolate strictly aerobic organisms, since these bacteria constitute only a small proportion of supragingival plaque flora, and as one of these organisms (viz. Neisseria) was cultured on some of the anaerobic plates, it was felt the above methods would be adequate.

Identification.

Following culture of plaque, attempts must be made to identify the organisms isolated. However some of the organisms are poorly characterised, and are therefore difficult to identify with confidence (Johnson & Murphy, 1983). The methods of identification used in studies have varied from crude morphological identification (Ostrom et al., 1977; Ellen et al., 1985), through physiological and biochemical tests, to more sophisticated techniques, such as electron microscopy, DNA probing and immunocytochemistry / immunofluorescence (Moore et al., 1982; Berthold & Listgarten, 1986). However, the most commonly used method in caries research is physiological and biochemical screening. It is accepted that this is not as accurate as the more sensitive methods named above, since the biochemical characteristics of different organisms are frequently very similar, but such screening methods allow large numbers of identifications to be made. Further, the more sophisticated equipment is not readily available.

In this thesis, bacterial identification was achieved using physiological and biochemical tests which were supplied as commercially available kits (see Section 2.5.5.). Unfortunately, there is considerable confusion at present concerning the taxonomy of the viridans streptococci, as there is lack of agreement on nomenclature, and current work suggests that some species should be reclassified and others subdivided (Kilian & Mikkelsen, 1986; Nyvad & Kilian, 1987). A new species of streptococcus (Strep. oralis) has been described in recent years (Bridge & Sneath, 1982), and it has been recommended that Strep. mitior and Strep. sanguis II should be included in this species (Kilpper - Bälz et al., 1985; Kilian & Mikkelsen, 1986). A recent taxonomic study of oral streptococci suggested that the names of Strep. mitior and Strep. mitis may have been substituted in error in the

API Strep. system, the identification system used in this study, and the authors recommended that Strep. mitis strains identified using the API system should be considered to be Strep. oralis. Furthermore they suggested that true Strep. mitis strains will probably be recorded in the API classification as Strep. sanguis I/1 and I/2 (Schmidhuber et al., 1987).

It was decided, therefore, in this study, to rename the organisms which had been identified as Strep. sanguis II and Strep. mitis, as Strep. oralis. However, from the results obtained using the API strips regarding the Strep. sanguis I/1 and I/2, it was not possible to separate Strep. mitis from Strep. sanguis I, and facilities for differentiating the organisms using the other criteria described by Schmidhuber and co-workers, were not available. Therefore, all organisms identified in this group are classified as Strep. sanguis. It is generally accepted that, at present, viridans streptococci cannot be identified unequivocably using only physiological and biochemical tests (Schmidhuber et al., 1987).

Problems are also encountered when using commercial kits in the identification of Gram positive and Gram negative rods. In particular, the tests may differentiate poorly between organisms at species, or even genus level. In the current study, the Minitek system was used for identification of positive bacilli as this was found to produce fewer reactions which were difficult to interpret. However, since the negative bacilli were often poorly discriminated in the Minitek system, these organisms were characterized using the API 20A system, although frequently, this was able to identify the organisms only to a genus level. Therefore, the results given throughout the study for positive and negative rods are mostly classified by genus alone.

Attempts were made, however, to subdivide Actinomyces into species, since this genus comprises a large proportion of the total cultivable flora in mature plaque samples. A. odontolyticus was readily differentiated from A. naeslundii and A. viscosus using cultural and biochemical data, but discrimination between the latter two species is more difficult. In the past, differentiation between them was based on the reaction to catalase, with positive organisms being classified as A. viscosus and those showing a negative reaction, as A. naeslundii (Ellen, 1976). However, taxonomic studies have shown that separation of the species on the basis of routine

physiological tests alone is inadequate (Fillery et al., 1978), and classification has therefore often been based on a combination of physiological, biochemical and serological testing (Hill et al., 1977; Ellen et al., 1985; Milnes & Bowden, 1985). Some authors have not attempted to separate the two species and have classified them together as A. viscosus / naeslundii (Keltjens et al., 1987). The discrimination between the two species using the Minitek system is based mainly on the catalase reaction, therefore in the current study the organisms have been classified as catalase positive or negative within the grouping A. viscosus / naeslundii.

In any biochemical method of identifying organisms, problems can arise from different investigators performing the tests in slightly varying manners. Thus results from different researchers may not be strictly comparable. However, while the shortcomings of the physiological and biochemical methods for identification of microorganisms are appreciated, they remain at present the only readily available and practicable means of identifying large numbers of plaque organisms, and for this reason, these techniques have been used in this study.

3.3. Site Variation.

3.3.1. Introduction.

Exogenously derived tooth specimens and synthetic materials are being employed increasingly for intra-oral studies in the field of caries research, since their use can often overcome ethical problems, allows easier accessibility to sites for plaque sampling, and permits the measurement of mineralisation changes.

Artificial supporting material such as Mylar strips, plastic films and epoxy resin, with and without the addition of hydroxyapatite (reviewed by Nyvad & Fejerskov, 1987), have all been used for studying the early stages of microbial colonisation in the oral cavity. While a study by Theilade and co-workers (1982) found no qualitative differences in the characterisation of three and eight hour plaque samples removed from Mylar strips and natural tooth surfaces, relatively few such studies have been undertaken to determine whether the results obtained using artificial materials are comparable with the natural situation, and the issue at present remains unresolved (Nyvad & Fejerskov, 1987).

Since the development of the intra-oral appliance model by Koulourides and co-workers in 1974, specimens of human or bovine tooth substance have increasingly been mounted on intra-oral devices, or implanted into restorations present in teeth, and used for a variety of investigations. These include testing the cariogenic potential of sugars and foods (Brudevold et al., 1988; Thomson, 1988), investigating the remineralising potential of fluoride-containing mouthrinses and dentifrices (Featherstone et al., 1982) and studying the microbiology of early colonisation of human enamel and root surfaces (Nyvad & Kilian, 1987) and occlusal fissures (Theilade et al., 1974).

Whilst tooth-derived specimens should resemble the natural situation more closely than artificial materials, the accessibility of saliva to these appliance-borne tooth components, the proximity of the sampling sites on them to the appliance material, and the method by which plaque is allowed to accumulate on their surface, may all affect the microbial flora associated with them.

The only previous study comparing microbial composition and acid anion profiles of plaque derived from natural tooth surfaces with plaque overlying enamel sections mounted on an appliance, was carried out by Creanor and co-workers (1986b), on one subject.

In this study, which involved five volunteers, a comparison was made of one-week old plaque samples removed from smooth surfaces of the natural dentition, appliance-borne human enamel sections and slabs, and from the acrylic base of the appliance. A comparison of acid-anion and pH profiles of plaque removed from enamel slabs, and from the natural tooth surface following a sucrose mouthrinse, was also carried out. In addition, this investigation was used to determine whether enamel demineralisation had occurred during the seven-day experimental period.

3.3.2. Experimental Protocol.

Five volunteers, three from Glasgow and two from Port Sunlight, were involved in this study. The Glaswegians each received a scale and polish, using a non-fluoridated paste, and the teeth of the lower arch were flossed immediately before the appliance was inserted in the mouth. However, since it was necessary to send the in situ appliances complete with sections and slabs to the two Port Sunlight volunteers, they were simply instructed to brush and floss the teeth of the lower arch immediately before placing the appliance in the mouth.

The trough areas of the appliances each contained an enamel section and four enamel slabs as shown in Fig 3.2.

The appliance was worn for one week, and was removed twice daily to allow toothbrushing of the subject's own dentition using a non-fluoridated dentifrice (Unilever Research, Port Sunlight, England). All regions of the mouth were cleaned in the normal manner except for the lingual and interproximal surfaces of the lower molars contiguous with the appliance, which were left untouched. The plaque which accumulated in the trough area of the appliance was also left undisturbed.

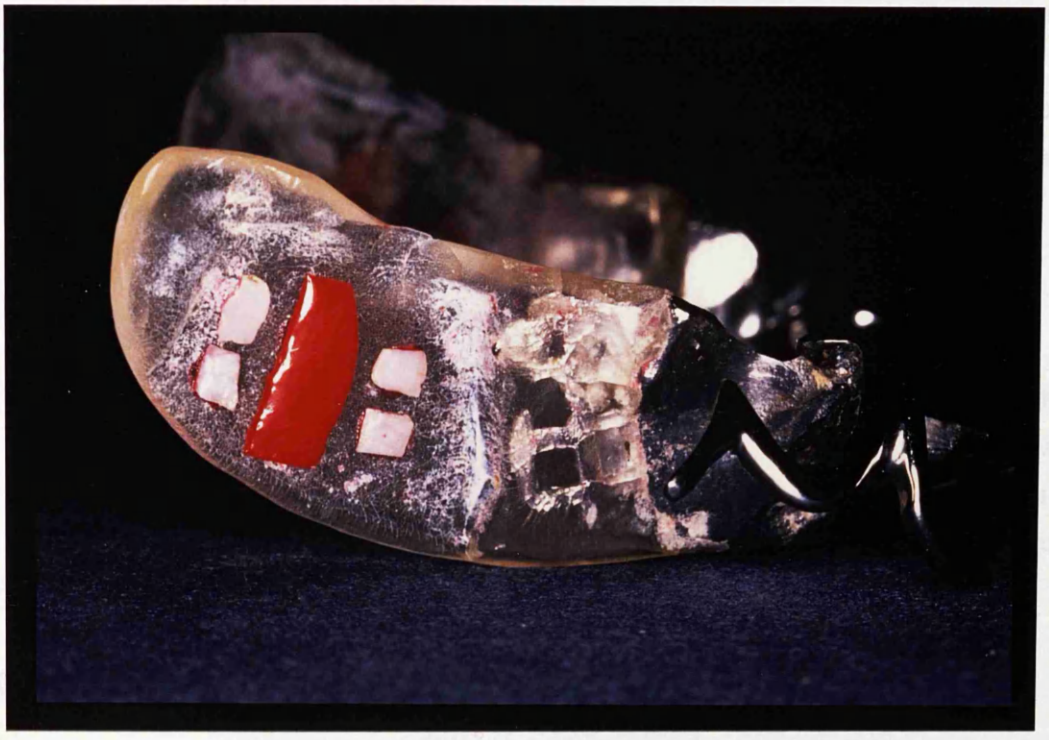


Fig. 3.2. **Trough area of appliance showing enamel section and four enamel slabs.**

3.3.3. Plaque Sampling.

At the end of one week, plaque samples were removed from the windows on the tooth sections (see 2.5.1.) and from the surface of two of the enamel slabs (described in 2.5.2.). Samples were also obtained from the area of acrylic on the base of the trough areas between the section and slabs, from the midpoint of the lingual surface of the lower first molar and from the distal surface of this tooth. Samples were obtained from left and right sides of both the appliance and natural dentition, using, in each case, a sterile dental excavator (No 243, Ash, England). Each sample was placed immediately in 1 ml ABB, and the methods described previously in 2.5.4. and 2.5.5. for culture and identification of the plaque microflora were followed.

3.3.4. Plaque Acid Anion and pH Profiles.

Plaque samples were also removed, using a dental excavator, from the lingual surface of the lower molars and from the remaining unsampled enamel slabs, both before and after a one minute 10 % sucrose mouthrinse, for acid anion estimations by isotachophoresis and plaque pH measurements as described in Sections 2.7.1. and 2.7.2.

3.3.5. Assessment of Demineralisation.

The enamel sections were radiographed and analysed by microdensitometry using the methods described in Section 2.4., both before and after the one week experimental period to assess whether or not demineralisation had occurred.

3.3.6. Results.

Effect of Subject on Microbial Flora.

The predominant cultivable flora isolated from plaque obtained from tooth surface, section, slab and acrylic, for each of the five subjects, is given in Tables 3.7. to 3.10. The column headings used in these tables are detailed in Table 3.7.

In the plaque obtained from the natural tooth surface, positive cocci were the most frequently isolated group of organisms, with the mean percentage of total anaerobic cultivable flora ranging, in the five subjects, from 41.2 to 58.6. The proportion of negative cocci was more variable, being from 5.7 to 18.8. Positive bacilli ranged from 15.8 to 44.0, and negative bacilli showed the greatest variation in proportion and isolation frequency - 0.7 % and an isolation frequency of 1 in 4, in subject A, to 20.2 % and an isolation frequency of 4 in 4 for subject E. Strep. mutans was isolated in none of the samples from 2 subjects, and had mean percentage counts of 3.1, 3.9 and 13.1 in the other subjects.

In plaque obtained from enamel sections, the mean proportion of positive cocci ranged from 58.6 % (Subject B) to 76.4 % (Subject C), while the corresponding values for negative cocci were 2.7 % (Subject A) and 23.2 % (Subject B). Positive bacilli ranged from a mean of 6.1 to 25.2 %, in the five subjects. Negative bacilli were undetected in Subject C, but rose to a mean proportion of 14.6 % of the total microflora in Subject D. Strep. mutans was only detected in Subject E, at a mean level of 3.5 %.

Enamel slab plaque again showed considerable variation in microbial composition in the five subjects, with the mean proportions of positive cocci ranging from 55.0 to 65.3 %, negative cocci varying from 3.8 to 16.5 %, positive bacilli rising from 14.0 to 27.2 %, and negative bacilli having a minimum mean percentage count of 0.9 % and a maximum of 14.8 %. Strep. mutans and lactobacilli were undetectable in all samples from each of the five subjects.

The mean proportion of positive cocci in plaque removed from acrylic ranged from 47.4 to 81.0 %, while negative cocci varied from 1.8 to 23.0 %. The lowest mean percentage count of positive bacilli was 5.6 %, and the highest 19.1 %, and the mean range for negative bacilli was ND - 19.8 %. Strep. mutans was detected in two out of four samples obtained from Subject E, and in no sample from any of the other four volunteers.

The statistical analysis shown in Table 3.16. confirms the differences between subjects, with the proportions of negative cocci and positive and negative bacilli all being subject dependent. Although no subject

difference was seen for positive cocci, the mean percentage counts of all three streptococcal species studied varied among the subjects ($p < 0.001$).

Effect of Surface on Microbial Flora.

The results for each surface, for all subjects combined, are given in Table 3.11. Significant differences between the surfaces (see Table 3.16.) were found in the percentages of positive cocci and positive bacilli isolated (both, $p < 0.001$). These differences were, in general, between plaque isolated from tooth surface and plaque isolated from each of the other three surfaces. For Strep. oralis there was a significant difference between slab and acrylic, but otherwise no significant differences in mean proportional counts of bacterial groups, genera or species were obtained, between section, slab and acrylic.

Three organisms which were found in low proportions were Fusobacterium, Capnocytophaga and Neisseria spp. Fusobacteria spp. were identified in plaque from 7/20 of tooth surfaces and 2/20 for each of the other surfaces, while Capnocytophaga spp. were isolated from 13/20 of tooth surfaces, and 8, 9 and 7 out of 20 sites for section, slab and acrylic surfaces respectively. Neisseria spp. were not found in any of the tooth surface plaques, although this organism was present in plaques from the other three surfaces, ranging from 2/20 - 6/20.

Thus, although the proportions of organisms identified from tooth surfaces varied from the other surfaces under examination, there was only the occasional difference in the range of organisms found.

Effect of Position on Microbial Flora.

To determine whether there was variation in the microflora isolated from different positions on each surface, the mean and standard deviation for each position on each surface, for all subjects combined, are shown in Tables 3.12. to 3.15.

The statistical analysis shown in Table 3.16. shows that, in general, the position was not significant, as only with Strep. mutans, in two positions, was there a significant position effect ($p < 0.05$).

Plaque pH and Acid Anion Profiles.

The pH response of plaque, present on natural tooth and enamel slab, to 10 % sucrose was measured for three subjects, and results shown in Table 3.17. In each case, the response to sucrose followed the pattern of a typical Stephan curve, as shown in Fig. 3.3., with the mean minimum pH being 5.54 (tooth) and 5.49 (enamel slab). Paired t-test analysis showed no significant difference between minimum pH or changes in pH from baseline to pH minimum (ΔpH) for the two surfaces.

In all five subjects, the acid response to sucrose, of plaque covering tooth and enamel slab, was measured, and the results are shown in Table 3.18. As the quantity of plaque removed in each case was very small, particularly in relation to the enamel slabs, the results are expressed as lactate / acetate ratio rather than concentration per mg wet weight of plaque. A paired t-test on the six minute lactate / acetate ratio, expressed as a proportion of the baseline ratio, showed no significant difference between the two surfaces.

Assessment of Demineralisation.

The enamel sections used in this study were examined by microradiography and microdensitometry at base-line and at the end of the seven day experimental period. In no case was there any evidence of demineralisation.

Table 3.7. % predominant cultivable microflora isolated from one week plaque obtained from natural tooth surfaces, in five Subjects, A - E, n=4.

TOOTH SUBJECT A

	F[a]	Mean[b] (SD)[c]	Median	Range
+ve cocci	4/4	46.4 (5.0)	45.5	42.2 - 52.4
-ve cocci	3/4	8.8 (12.5)	3.9	ND[d] - 27.3
+ve bacilli	4/4	44.0 (10.3)	45.7	30.3 - 54.5
-ve bacilli	1/4	0.7 (1.3)	ND	ND - 2.7
S. mutans	3/4	3.1 (4.2)	1.6	ND - 9.1
S. sanguis	4/4	24.9 (3.8)	24.0	21.2 - 30.3
S. oralis	3/4	17.0 (12.6)	19.8	ND - 28.6
Veillonella	3/4	8.8 (12.5)	3.9	ND - 27.3
Actinomyces	3/4	44.0 (10.3)	45.7	30.3 - 54.5
Lactobacillus	0/4	ND	ND	ND
Bacteroides	1/4	0.7 (1.4)	ND	ND - 2.7

TOOTH SUBJECT B

	F	Mean (SD)	Median	Range
+ve cocci	4/4	41.4 (8.9)	38.2	35.0 - 54.5
-ve cocci	3/4	13.7 (10.6)	15.0	ND - 25.0
+ve bacilli	4/4	27.3 (19.3)	20.7	12.1 - 55.6
-ve bacilli	4/4	17.5 (6.8)	20.6	7.4 - 21.4
S. mutans	0/4	ND	ND	ND
S. sanguis	3/4	17.7 (12.1)	21.8	ND - 27.3
S. oralis	4/4	19.0 (7.0)	18.9	10.8 - 27.3
Veillonella	3/4	14.6 (11.1)	16.8	ND - 25.0
Actinomyces	4/4	27.3 (19.3)	20.7	12.1 - 55.6
Lactobacillus	0/4	ND	ND	ND
Bacteroides	3/4	9.6 (6.9)	11.7	ND - 15.0

[a] - Isolation frequency ; [b] - Mean percentage of total cfu ;
[c] - Standard deviation ; [d] - Not detected.

TOOTH
SUBJECT C

	F	Mean (SD)	Median	Range
+ve cocci	4/4	58.6 (28.3)	48.0	38.1 -100.0
-ve cocci	3/4	18.8 (13.1)	22.4	ND - 30.4
+ve bacilli	3/4	17.8 (12.4)	21.4	ND - 28.6
-ve bacilli	3/4	4.8 (3.9)	4.8	ND - 9.5
S. mutans	2/4	3.9 (4.9)	2.4	ND - 10.5
S. sanguis	4/4	20.7 (11.4)	18.4	10.5 - 35.5
S. oralis	4/4	23.3 (27.5)	10.0	8.7 - 64.5
Veillonella	3/4	18.8 (13.1)	22.4	ND - 30.4
Actinomyces	3/4	16.5 (12.2)	18.8	ND - 28.6
Lactobacillus	1/4	1.4 (2.6)	ND	ND - 5.3
Bacteroides	0/4	ND	ND	ND

TOOTH
SUBJECT D

	F	Mean (SD)	Median	Range
+ve cocci	4/4	41.2 (7.0)	38.3	36.4 - 51.7
-ve cocci	4/4	5.7 (3.0)	4.6	3.4 - 10.2
+ve bacilli	4/4	41.5 (13.0)	43.8	23.8 - 54.5
-ve bacilli	4/4	11.6 (14.5)	4.8	3.4 - 33.3
S. mutans	0/4	ND	ND	ND
S. sanguis	3/4	19.0 (12.8)	24.2	ND - 27.6
S. oralis	3/4	12.6 (9.9)	13.2	ND - 24.1
Veillonella	4/4	5.7 (3.0)	4.6	3.4 - 10.2
Actinomyces	4/4	35.7 (14.5)	41.2	14.3 - 46.2
Lactobacillus	2/4	3.5 (4.4)	2.4	ND - 9.1
Bacteroides	1/4	6.0 (11.9)	ND	ND - 23.8

Table 3.7. cont.

TOOTH
SUBJECT E

	F	Mean (SD)	Median	Range
+ve cocci	4/4	57.8 (16.1)	57.4	40.0 - 76.4
-ve cocci	1/4	6.2 (9.1)	2.7	ND - 19.2
+ve bacilli	4/4	15.8 (10.5)	13.8	5.4 - 30.0
-ve bacilli	4/4	20.2 (7.2)	19.0	12.7 - 30.0
S. mutans	3/4	13.1 (10.1)	14.0	ND - 24.3
S. sanguis	4/4	21.6 (8.6)	21.0	13.5 - 30.9
S. oralis	4/4	17.4 (8.3)	18.6	7.7 - 25.0
Veillonella	1/4	6.2 (9.1)	2.7	ND - 19.2
Actinomyces	4/4	15.8 (10.5)	13.8	5.4 - 30.0
Lactobacillus	0/4	ND	ND	ND
Bacteroides	4/4	5.2 (3.9)	4.2	1.8 - 10.8

Table 3.7. cont.

Table 3.8. % predominant cultivable microflora isolated from one week plaque obtained from enamel sections, in five Subjects, A - E, n=4.

SECTION SUBJECT A

	F	Mean (SD)	Median	Range
+ve cocci	4/4	68.3 (12.2)	66.2	57.7 - 83.3
-ve cocci	3/4	2.7 (2.0)	3.1	ND - 4.5
+ve bacilli	4/4	25.2 (7.6)	34.6	16.7 - 34.6
-ve bacilli	3/4	3.8 (3.9)	3.1	ND - 9.1
S. mutans	0/4	ND	ND	ND
S. sanguis	4/4	36.8 (20.3)	29.4	22.0 - 66.7
S. oralis	4/4	31.5 (14.5)	29.0	16.7 - 51.3
Veillonella	3/4	2.7 (2.0)	3.1	ND - 4.5
Actinomyces	4/4	25.2 (7.6)	24.6	16.7 - 34.6
Lactobacillus	0/4	ND	ND	ND
Bacteroides	1/4	2.3 (4.6)	ND	ND - 9.1

SECTION SUBJECT B

	F	Mean (SD)	Median	Range
+ve cocci	4/4	58.6 (12.0)	58.4	45.4 - 71.9
-ve cocci	4/4	23.2 (6.7)	22.6	15.6 - 31.8
+ve bacilli	4/4	11.8 (7.2)	10.6	4.5 - 21.7
-ve bacilli	3/4	6.4 (8.1)	3.7	ND - 18.2
S. mutans	0/4	ND	ND	ND
S. sanguis	4/4	31.8 (20.2)	36.4	4.5 - 50.0
S. oralis	4/4	26.7 (14.4)	28.6	8.7 - 40.9
Veillonella	4/4	21.7 (7.2)	19.6	15.6 - 31.8
Actinomyces	4/4	10.4 (7.8)	7.6	4.5 - 21.7
Lactobacillus	1/4	1.5 (3.0)	ND	ND - 5.9
Bacteroides	1/4	2.3 (4.6)	ND	ND - 9.1

SECTION
SUBJECT C

	F	Mean (SD)	Median	Range
+ve cocci	4/4	76.4 (5.1)	76.2	70.6 - 82.8
-ve cocci	3/3	10.6 (7.3)	12.8	ND - 16.7
+ve bacilli	4/4	13.0 (8.7)	13.0	3.4 - 22.7
-ve bacilli	0/4	ND	ND	ND
S. mutans	0/4	ND	ND	ND
S. sanguis	4/4	27.3 (12.3)	27.6	12.5 - 41.4
S. oralis	4/4	40.9 (6.4)	41.6	33.3 - 47.0
Veillonella	3/4	8.8 (7.1)	9.4	ND - 16.7
Actinomyces	4/4	13.0 (8.7)	13.0	3.4 - 22.7
Lactobacillus	0/4	ND	ND	ND
Bacteroides	0/4	ND	ND	ND

SECTION
SUBJECT D

	F	Mean (SD)	Median	Range
+ve cocci	4/4	65.5 (4.5)	65.5	60.0 - 71.0
-ve cocci	4/4	13.8 (6.6)	14.6	6.2 - 19.4
+ve bacilli	4/4	6.1 (4.3)	4.4	3.2 - 12.5
-ve bacilli	4/4	14.6 (7.9)	13.6	6.4 - 25.0
S. mutans	0/4	ND	ND	ND
S. sanguis	4/4	55.3 (4.6)	55.6	50.0 - 60.0
S. oralis	2/4	5.6 (6.5)	4.8	ND - 12.5
Veillonella	4/4	13.7 (6.6)	14.6	6.2 - 19.4
Actinomyces	4/4	6.1 (4.3)	4.4	3.2 - 12.5
Lactobacillus	0/4	ND	ND	ND
Bacteroides	3/4	10.5 (9.0)	11.0	ND - 20.0

Table 3.8. cont.

SECTION
SUBJECT E

	F	Mean (SD)	Median	Range
+ve cocci	4/4	66.9 (13.3)	62.9	55.8 - 86.1
-ve cocci	4/4	16.8 (9.8)	19.4	2.8 - 25.6
+ve bacilli	3/4	9.1 (8.2)	9.3	ND - 17.8
-ve bacilli	4/4	7.2 (3.6)	7.0	3.6 - 11.1
S. mutans	1/4	3.5 (7.0)	ND	ND - 14.0
S. sanguis	3/4	14.0 (10.0)	16.4	ND - 23.2
S. oralis	4/4	45.2 (27.3)	36.0	25.5 - 83.4
Veillonella	4/4	16.8 (9.8)	19.4	2.8 - 25.6
Actinomyces	3/4	9.1 (8.2)	9.3	ND - 17.8
Lactobacillus	0/4	ND	ND	ND
Bacteroides	0/4	ND	ND	ND

Table 3.8. cont.

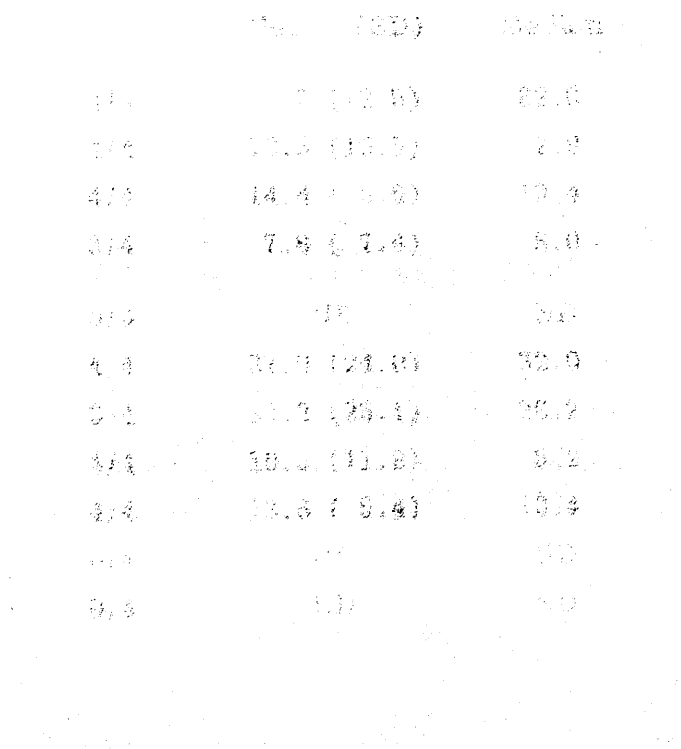


Table 3.9. % predominant cultivable microflora isolated from one week plaque obtained from enamel slabs, in five Subjects, A - E, n=4.

SLAB
SUBJECT A

	F	Mean (SD)	Median	Range
+ve cocci	4/4	63.7 (29.9)	58.6	37.5 -100.0
-ve cocci	3/4	3.8 (3.5)	3.6	ND - 8.3
+ve bacilli	3/4	27.2 (24.4)	29.3	ND - 50.0
-ve bacilli	3/4	5.3 (4.1)	6.0	ND - 9.1
S. mutans	0/4	ND	ND	ND
S. sanguis	3/4	21.6 (26.6)	13.0	ND - 60.5
S. oralis	4/4	36.0 (27.1)	26.1	15.8 - 76.0
Veillonella	3/4	3.8 (3.5)	3.6	ND - 8.3
Actinomyces	3/4	26.1 (23.2)	29.3	ND - 45.8
Lactobacillus	0/4	ND	ND	ND
Bacteroides	3/4	5.3 (4.1)	6.0	ND - 9.1

SLAB
SUBJECT B

	F	Mean (SD)	Median	Range
+ve cocci	4/4	65.3 (12.5)	69.6	46.9 - 75.0
-ve cocci	4/4	12.5 (10.5)	7.9	6.1 - 28.1
+ve bacilli	4/4	14.4 (6.5)	13.4	8.7 - 21.9
-ve bacilli	3/4	7.8 (7.4)	8.0	ND - 15.2
S. mutans	0/4	ND	ND	ND
S. sanguis	4/4	37.0 (24.0)	32.0	14.3 - 69.7
S. oralis	3/4	28.2 (25.1)	26.2	ND - 60.7
Veillonella	4/4	10.5 (11.8)	5.2	3.6 - 28.1
Actinomyces	4/4	13.6 (5.4)	13.4	8.7 - 18.8
Lactobacillus	0/4	ND	ND	ND
Bacteroides	0/4	ND	ND	ND

SLAB
SUBJECT C

	F	Mean (SD)	Median	Range
+ve cocci	4/4	56.0 (17.4)	58.4	36.1 - 75.0
-ve cocci	4/4	16.3 (4.5)	14.1	13.9 - 23.1
+ve bacilli	4/4	25.8 (19.0)	21.6	10.2 - 50.0
-ve bacilli	1/4	0.9 (1.8)	ND	ND - 3.6
S. mutans	0/4	ND	ND	ND
S. sanguis	4/4	33.0 (9.1)	32.7	22.2 - 44.4
S. oralis	4/4	23.3 (8.7)	24.2	13.9 - 30.8
Veillonella	4/4	16.3 (4.5)	14.1	13.9 - 23.1
Actinomyces	4/4	25.8 (19.0)	21.6	10.2 - 50.0
Lactobacillus	0/4	ND	ND	ND
Bacteroides	0/4	ND	ND	ND

SLAB
SUBJECT D

	F	Mean (SD)	Median	Range
+ve cocci	4/4	55.0 (4.4)	56.0	48.8 - 59.1
-ve cocci	4/4	16.5 (13.1)	15.9	2.6 - 31.7
+ve bacilli	4/4	16.8 (2.6)	17.0	13.6 - 19.5
-ve bacilli	3/4	11.7 (11.8)	10.2	ND - 26.3
S. mutans	0/4	ND	ND	ND
S. sanguis	4/4	51.9 (4.7)	52.2	46.3 - 56.8
S. oralis	2/4	1.9 (2.5)	1.2	ND - 5.3
Veillonella	4/4	16.5 (13.1)	15.9	2.6 - 31.7
Actinomyces	4/4	16.2 (2.0)	16.4	13.6 - 18.2
Lactobacillus	0/4	ND	ND	ND
Bacteroides	3/4	6.0 (4.8)	6.8	ND - 10.5

Table 3.9. cont.

SLAB
SUBJECT E

	F	Mean (SD)	Median	Range
+ve cocci	4/4	57.8 (6.9)	58.6	50.0 - 63.9
-ve cocci	3/4	13.4 (15.2)	9.6	ND - 34.6
+ve bacilli	4/4	14.0 (8.7)	11.0	7.7 - 26.3
-ve bacilli	4/4	14.8 (15.4)	8.0	5.3 - 37.8
S. mutans	0/4	ND	ND	ND
S. sanguis	4/4	34.3 (24.7)	32.6	8.1 - 63.9
S. oralis	2/4	16.7 (21.8)	10.5	ND - 45.9
Veillonella	3/4	11.4 (15.7)	5.4	ND - 34.6
Actinomyces	4/4	14.0 (8.7)	11.0	7.7 - 26.3
Lactobacillus	0/4	ND	ND	ND
Bacteroides	0/4	ND	ND	ND

Table 3.9. cont.

Table 3.10. % predominant cultivable microflora isolated from one week plaque obtained from acrylic surfaces, in five Subjects, A - E, n=4.

ACRYLIC SUBJECT A

	F	Mean (SD)	Median	Range
+ve cocci	4/4	81.0 (14.1)	82.0	63.6 - 96.3
-ve cocci	2/4	1.8 (2.2)	1.4	ND - 4.5
+ve bacilli	4/4	16.0 (10.2)	16.6	3.7 - 27.3
-ve bacilli	1/4	1.1 (2.2)	ND	ND - 4.5
S. mutans	0/4	ND	ND	ND
S. sanguis	4/4	32.8 (17.2)	30.3	14.5 - 55.9
S. oralis	4/4	49.3 (25.5)	47.5	20.6 - 81.5
Veillonella	2/4	1.8 (2.2)	1.4	ND - 4.5
Actinomyces	4/4	16.0 (10.2)	16.6	3.7 - 27.3
Lactobacillus	0/4	ND	ND	ND
Bacteroides	1/4	1.1 (2.2)	ND	ND - 4.5

ACRYLIC SUBJECT B

	F	Mean (SD)	Median	Range
+ve cocci	4/4	65.6 (6.9)	67.2	56.7 - 71.4
-ve cocci	4/4	23.0 (3.9)	23.8	17.8 - 26.7
+ve bacilli	4/4	5.6 (3.0)	4.4	3.6 - 10.0
-ve bacilli	3/4	5.7 (4.0)	6.9	ND - 9.1
S. mutans	0/4	ND	ND	ND
S. sanguis	4/4	21.6 (16.1)	19.0	6.7 - 41.7
S. oralis	4/4	44.1 (14.0)	43.2	29.2 - 60.7
Veillonella	4/4	16.9 (6.4)	17.6	9.1 - 23.3
Actinomyces	4/4	4.8 (1.4)	4.4	3.6 - 6.7
Lactobacillus	0/4	ND	ND	ND
Bacteroides	1/4	1.1 (2.2)	ND	ND - 4.5

ACRYLIC SUBJECT C

	F	Mean (SD)	Median	Range
+ve cocci	4/4	68.7 (12.3)	68.2	55.0 - 83.3
-ve cocci	4/4	12.2 (5.3)	10.2	8.3 - 20.0
+ve bacilli	4/4	19.1 (13.8)	17.3	6.7 - 35.0
-ve bacilli	0/4	ND	ND	ND
S. mutans	0/4	ND	ND	ND
S. sanguis	4/4	26.8 (14.4)	26.6	10.5 - 43.3
S. oralis	4/4	32.7 (14.1)	32.5	15.8 - 50.0
Veillonella	4/4	11.4 (3.7)	10.2	8.3 - 16.7
Actinomyces	4/4	16.5 (11.0)	14.6	6.7 - 30.0
Lactobacillus	1/4	1.3 (2.4)	ND	ND - 5.0
Bacteroides	0/4	ND	ND	ND

ACRYLIC SUBJECT D

	F	Mean (SD)	Median	Range
+ve cocci	4/4	71.4 (14.1)	72.6	56.2 - 86.2
-ve cocci	4/4	13.2 (10.6)	10.7	3.4 - 28.1
+ve bacilli	4/4	11.0 (1.4)	11.2	9.4 - 12.5
-ve bacilli	2/4	3.8 (5.7)	1.6	ND - 12.0
S. mutans	0/4	ND	ND	ND
S. sanguis	4/4	60.8 (3.4)	60.8	56.2 - 64.0
S. oralis	2/4	9.9 (12.0)	7.8	ND - 24.1
Veillonella	4/4	13.2 (10.6)	10.7	3.4 - 28.1
Actinomyces	4/4	11.0 (1.4)	11.2	9.4 - 12.5
Lactobacillus	0/4	ND	ND	ND
Bacteroides	1/4	3.0 (6.0)	ND	ND - 12.0

Table 3.10. cont.

ACRYLIC
SUBJECT E

	F	Mean (SD)	Median	Range
+ve cocci	4/4	47.4 (20.2)	53.2	19.4 - 63.6
-ve cocci	4/4	17.6 (7.8)	18.0	9.1 - 25.0
+ve bacilli	3/4	15.3 (15.1)	12.9	ND - 35.5
-ve bacilli	4/4	19.8 (8.6)	17.2	12.5 - 32.2
S. mutans	2/4	4.7 (6.7)	2.2	ND - 14.3
S. sanguis	2/4	3.6 (6.0)	0.9	ND - 12.5
S. oralis	4/4	37.6 (17.2)	36.0	19.4 - 59.1
Veillonella	4/4	16.5 (6.6)	16.8	9.1 - 23.2
Actinomyces	3/4	13.7 (12.3)	12.9	ND - 29.0
Lactobacillus	1/4	1.7 (3.2)	ND	ND - 6.4
Bacteroides	3/4	4.1 (3.7)	3.7	ND - 8.9

Table 3.10. cont.

Table 3.11. % predominant cultivable microflora isolated from one week plaque obtained from each surface, for all Subjects combined, n=20.

TOOTH
ALL SUBJECTS

	F	Mean (SD)	Median	Range
+ve cocci	20/20	49.1 (15.9)	43.0	35.0 -100.0
-ve cocci	14/20	10.6 (10.5)	5.1	ND - 30.4
+ve bacilli	19/20	29.3 (17.0)	26.2	ND - 55.6
-ve bacilli	16/20	10.9 (10.4)	6.4	ND - 33.3
S. mutans	8/20	4.0 (6.9)	ND	ND - 24.3
S. sanguis	17/20	20.8 (9.5)	23.2	ND - 35.5
S. oralis	18/20	17.9 (13.8)	14.4	ND - 64.5
Veillonella	14/20	10.6 (10.5)	5.1	ND - 30.4
Actinomyces	18/20	27.9 (16.6)	25.2	ND - 55.6
Lactobacillus	3/20	1.0 (2.5)	ND	ND - 9.1
Bacteroides	9/20	4.3 (6.8)	ND	ND - 23.8

SECTION
ALL SUBJECTS

	F	Mean (SD)	Median	Range
+ve cocci	20/20	67.1 (10.8)	65.6	45.4 - 86.1
-ve cocci	18/20	13.4 (9.3)	14.7	ND - 31.8
+ve bacilli	19/20	13.0 (9.3)	12.2	ND - 34.6
-ve bacilli	14/20	6.4 (7.0)	4.0	ND - 25.0
S. mutans	9/20	0.7 (3.1)	ND	ND - 14.0
S. sanguis	19/20	33.1 (19.1)	30.6	ND - 66.7
S. oralis	18/20	30.0 (20.0)	29.0	ND - 83.4
Veillonella	18/20	12.7 (9.2)	13.7	ND - 31.8
Actinomyces	19/20	12.8 (9.5)	11.0	ND - 34.6
Lactobacillus	1/20	0.3 (1.3)	ND	ND - 5.9
Bacteroides	5/20	3.0 (5.9)	ND	ND - 20.0

SLAB
ALL SUBJECTS

	F	Mean (SD)	Median	Range
+ve cocci	20/20	59.7 (15.5)	58.0	36.1 -100.0
-ve cocci	18/20	12.5 (10.4)	8.9	ND - 34.6
+ve bacilli	19/20	19.6 (14.3)	14.8	ND - 50.0
-ve bacilli	14/20	8.1 (9.8)	4.9	ND - 37.8
S. mutans	0/20	ND	ND	ND
S. sanguis	19/20	35.6 (20.4)	36.2	ND - 69.7
S. oralis	15/20	21.2 (21.1)	19.4	ND - 76.0
Veillonella	18/20	11.7 (10.8)	7.2	ND - 34.6
Actinomyces	19/20	19.1 (13.9)	14.8	ND - 50.0
Lactobacillus	0/20	ND	ND	ND
Bacteroides	6/20	2.3 (3.8)	ND	ND - 10.5

ACRYLIC
ALL SUBJECTS

	F	Mean (SD)	Median	Range
+ve cocci	20/20	66.9 (16.9)	63.8	19.4 - 96.3
-ve cocci	18/20	13.6 (9.3)	11.2	ND - 28.1
+ve bacilli	19/20	13.4 (10.4)	10.2	ND - 35.5
-ve bacilli	10/20	6.1 (8.6)	1.6	ND - 32.2
S. mutans	2/20	1.0 (3.3)	ND	ND - 14.4
S. sanguis	18/20	29.0 (22.1)	27.3	ND - 64.0
S. oralis	18/20	34.7 (20.7)	32.5	ND - 81.5
Veillonella	18/20	12.0 (8.1)	10.2	ND - 28.1
Actinomyces	19/20	12.4 (8.9)	9.8	ND - 30.0
Lactobacillus	2/20	0.6 (1.8)	ND	ND - 6.4
Bacteroides	6/20	1.9 (3.4)	ND	ND - 12.0

Table 3.11. cont.

TOOTH
ALL SUBJECTS

	L.lingual Mean (SD)	L.proximal Mean (SD)	R.lingual Mean (SD)	R.proximal Mean (SD)	All sites Mean (SD)
+ve cocci	45.2(11.3)	52.8(14.9)	46.3(7.0)	52.0(27.3)	49.1(15.9)
-ve cocci	8.7(12.8)	14.1(9.8)	13.8(9.1)	5.9(10.9)	10.6(10.5)
+ve bacilli	38.8(18.6)	18.5(9.8)	29.1(13.3)	30.6(22.0)	29.3(17.0)
-ve bacilli	7.1(7.1)	14.5(13.2)	10.7(9.4)	11.4(13.0)	11.0(10.4)
S. mutans	5.5(10.6)	7.2(7.1)	3.3(5.0)	ND	4.0(6.9)
S. sanguis	19.1(5.6)	19.8(13.9)	24.7(2.5)	19.5(13.1)	20.8(9.5)
S. oralis	12.2(2.5)	12.3(12.8)	17.5(9.0)	29.5(20.1)	17.9(13.8)
Veillonella	8.7(12.8)	14.1(9.8)	14.2(9.7)	5.9(10.9)	10.8(10.6)
Actinomyces	38.8(18.6)	15.6(9.1)	29.1(13.3)	27.9(19.0)	27.9(16.6)
Lactobacillus	ND	2.0(2.8)	ND	1.8(4.1)	1.0(2.5)
Bacteroides	2.2(4.8)	6.9(10.1)	3.5(6.2)	4.5(6.2)	4.3(6.8)

Table 3.12. % predominant cultivable microflora isolated from one week plaque samples from each site on natural tooth surface, for all Subjects combined, n=5.

SECTION
ALL SUBJECTS

	L.superior Mean (SD)	L.inferior Mean (SD)	R.superior Mean (SD)	R.inferior Mean (SD)	All sites Mean (SD)
+ve cocci	75.4(6.1)	67.2(9.3)	65.7(13.8)	68.2(9.4)	67.1(10.8)
-ve cocci	11.4(8.1)	13.6(8.7)	11.5(11.9)	17.0(10.1)	13.4(9.3)
+ve bacilli	8.6(8.4)	13.4(12.6)	16.0(7.1)	14.2(10.0)	13.0(9.4)
-ve bacilli	4.6(4.3)	5.7(6.7)	6.8(10.4)	8.5(7.1)	6.4(7.0)
S. mutans	ND	2.8(6.2)	ND	ND	0.7(3.1)
S. sanguis	28.5(24.7)	34.8(12.3)	44.1(19.8)	24.8(17.4)	33.1(19.1)
S. oralis	39.9(28.7)	28.4(10.0)	19.3(17.4)	32.3(19.7)	30.0(20.0)
Veillonella	11.4(8.1)	11.1(7.6)	11.5(11.9)	17.0(10.1)	12.7(9.2)
Actinomyces	8.6(8.4)	12.2(13.0)	16.0(7.1)	14.2(10.0)	12.8(9.5)
Lactobacillus	ND	1.2(2.6)	ND	ND	0.3(1.3)
Bacteroides	1.3(2.9)	3.1(7.0)	4.0(8.9)	3.6(5.0)	3.0(5.9)

Table 3.13. % predominant cultivable microflora isolated from one week plaque samples from each site on enamel sections, for all Subjects combined, n=5.

SLAB
ALL SUBJECTS

	L.superior Mean (SD)	L.inferior Mean (SD)	R.superior Mean (SD)	R.inferior Mean (SD)	All sites Mean (SD)
+ve cocci	64.7(11.1)	54.4(14.9)	62.2(24.3)	57.6(11.4)	59.7(15.5)
-ve cocci	10.4(8.1)	19.2(10.1)	11.4(14.0)	9.1(8.7)	12.5(10.4)
+ve bacilli	20.6(8.3)	23.3(15.6)	16.5(19.5)	18.1(15.8)	19.6(14.3)
-ve bacilli	4.3(2.9)	3.1(3.4)	9.8(11.2)	15.2(14.0)	8.1(9.8)
S. mutans	ND	ND	ND	ND	ND
S. sanguis	36.5(20.3)	38.4(20.0)	37.2(26.8)	30.2(19.8)	35.6(20.4)
S. oralis	23.1(22.5)	16.0(13.4)	19.0(32.3)	26.9(16.7)	21.2(21.1)
Veillonella	9.7(8.6)	17.5(11.8)	11.4(14.0)	8.2(8.9)	11.7(10.8)
Actinomyces	20.6(8.3)	21.3(14.0)	16.5(19.5)	18.1(15.8)	19.1(13.9)
Lactobacillus	ND	ND	ND	ND	ND
Bacteroides	2.5(3.6)	0.8(1.9)	2.1(4.7)	3.6(5.0)	2.3(3.8)

Table 3.14. % predominant cultivable microflora isolated from one week plaque samples from each enamel slab position, for all Subjects combined, n=5.

ACRYLIC
ALL SUBJECTS

	L.superior Mean (SD)	L.inferior Mean (SD)	R.superior Mean (SD)	R.inferior Mean (SD)	All sites Mean (SD)
+ve cocci	59.6(11.5)	67.8(12.3)	78.0(13.1)	62.2(25.7)	66.9(16.9)
-ve cocci	15.3(10.2)	15.9(11.0)	11.5(10.7)	11.6(6.9)	13.6(9.3)
+ve bacilli	18.9(9.9)	11.0(10.2)	6.8(2.9)	17.0(13.6)	13.4(10.4)
-ve bacilli	6.2(6.1)	5.3(6.7)	3.6(8.1)	9.2(13.4)	6.1(8.6)
S. mutans	0.9(1.9)	2.9(6.4)	ND	ND	1.0(3.3)
S. sanguis	31.8(26.3)	22.5(22.2)	32.4(24.7)	29.5(21.1)	29.0(22.1)
S. oralis	27.0(18.5)	34.7(25.9)	44.8(24.7)	32.5(14.6)	34.7(20.7)
Veillonella	13.8(8.3)	15.2(11.0)	10.0(8.7)	8.8(3.0)	12.0(8.1)
Actinomyces	17.2(8.8)	9.9(8.3)	6.8(2.9)	15.7(11.5)	12.4(8.0)
Lactobacillus	1.0(2.2)	ND	ND	1.3(2.9)	0.6(1.8)
Bacteroides	3.2(5.2)	1.8(4.0)	ND	2.4(2.3)	1.9(3.4)

Table 3.15. % predominant cultivable microflora isolated from one week plaque samples from each acrylic position, for all Subjects combined, n=5.

	Surface	Position	Subject
+ve cocci	***	NS	NS
-ve cocci	NS	NS	**
+ve bacilli	***	NS	*
-ve bacilli	NS	NS	***
S. mutans	**	*	***
S. sanguis	*	NS	***
S. oralis	**	NS	***
Veillonella	NS	NS	**
Actinomyces	***	NS	*
Lactobacillus	NS	NS	NS
Bacteroides	NS	NS	*

NS = Not significant ; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Table 3.16. Statistical analysis of effect of surface, position and subject on % predominant cultivable plaque microflora.

TOOTH SURFACE PLAQUE

	plaque pH response			
Time (min)	Subject A	Subject B	Subject C	Mean (SD)
0	7.35	7.17	7.52	7.35 (0.18)
1	5.87	6.10	5.91	5.96 (0.12)
3	5.43	5.58	5.62	5.54 (0.10)
6	5.11	5.49	6.01	5.54 (0.45)
10	5.67	5.93	6.58	6.06 (0.47)
15	6.36	6.42	7.20	6.66 (0.47)
20	6.53	6.63	7.40	6.85 (0.48)
30	6.75	7.03	7.42	7.07 (0.34)

ENAMEL SLAB PLAQUE

	plaque pH response			
Time (min)	Subject A	Subject B	Subject C	Mean (SD)
0	7.23	7.24	7.16	7.21 (0.04)
1	5.85	6.30	6.43	6.19 (0.30)
3	5.47	5.51	5.54	5.51 (0.04)
6	5.24	5.45	5.79	5.49 (0.28)
10	5.59	5.97	5.87	5.81 (0.20)
15	6.27	6.51	7.25	6.68 (0.51)
20	6.61	6.72	7.42	6.92 (0.44)
30	6.80	6.95	7.48	7.08 (0.36)

Table 3.17. Tooth surface and enamel slab plaque pH response to 10 % sucrose application in three subjects.

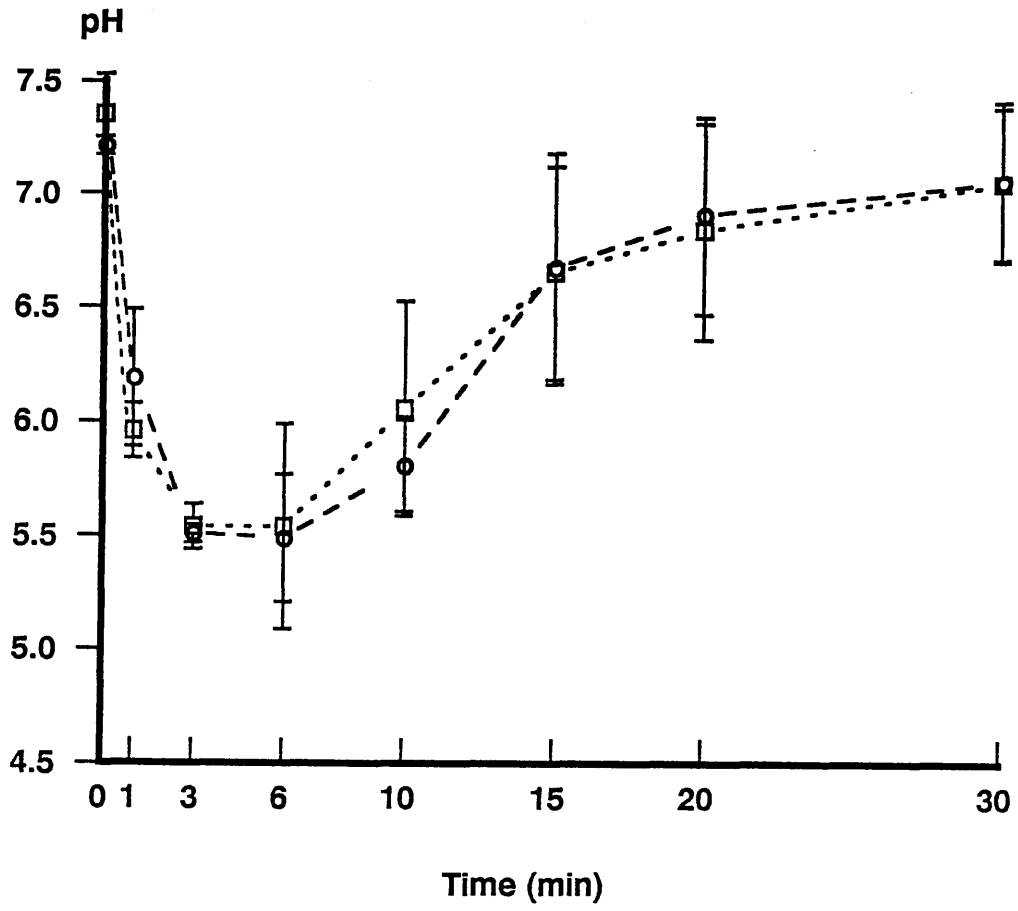


Fig. 3.3 **Mean (SD) pH response of natural tooth (square) and enamel slab (circle) plaque to 10 % sucrose mouthrinse, n=3.**

LACTATE / ACETATE RATIO

Subject	Tooth		Slab	
	0	6 min	0	6 min
A	0.18 (0.09)	1.54 (1.02)	0.23 (0.04)	1.49 (0.79)
B	0.17 (0.14)	0.84 (0.19)	0.22 (0.08)	0.90 (0.11)
C	0.24 (0.18)	0.66 (0.57)	0.20 (0.08)	0.72 (0.08)
D	0.28 (0.16)	0.94 (0.44)	0.48 (0.42)	1.30 (0.60)
E	0.32 (0.02)	1.82 (0.50)	0.30 (0.18)	1.72 (0.30)
All	0.25 (0.15)	1.16 (0.65)	0.28 (0.19)	1.23 (0.52)

Table 3.18. Mean (SD) of tooth surface and enamel slab plaque lactate / acetate ratio, before and 6 min after 10 % sucrose application, n=3.

3.3.7. Discussion.

Plaque Microflora.

This study shows significant differences between five subjects in the proportions of organisms in the total anaerobic cultivable flora from one week old plaque, retrieved from four surfaces. This finding of inter-subject differences in the composition of plaque microflora is in agreement with findings from other studies (Bowden et al., 1975), and is not surprising considering the variation in plaque composition which has been shown within subjects, and even from adjacent sites on the same tooth (Marsh & Keevil, 1986b).

Samples of one week plaque were removed from two lingual and two interproximal regions of the lower molars of the five subjects. While there was a little variation between the lingual and inter-proximal samples, overall no significant difference was found between the microflora of plaque removed from the two sites. The similarity of the two plaques may reflect the fact that a small dental excavator was used for all plaque sampling, and this may not have allowed access to deep inter-proximal regions, where plaque containing a higher anaerobic flora may have been expected (Marsh & Keevil, 1986b).

The mean percentages of the total cultivable microflora from the natural tooth surfaces is similar to the composition of one week supragingival plaque reported in other studies, with the Gram positive cocci predominating in all cases, followed by Gram positive bacilli, with negative cocci and bacilli comprising a smaller proportion of the total (Ritz, 1967; Syed & Loesche, 1978; van Palenstein Helderman, 1981).

A study on the predominant cultivable flora of plaque obtained from the fitting surface of acrylic dentures (Theilade et al., 1983) found that, in eight subjects, the mean percentage of positive cocci was approximately 50 %, positive bacilli 35 %, negative cocci 10 % and negative bacilli 1 %. The mean proportions of organisms in plaque removed from the acrylic of the appliance adjacent to the mounted enamel specimens, in the present study, found that positive cocci were higher (66.9%) and positive bacilli

lower (13.4 %), although the ranges for these groups of organisms are within the ranges quoted in the former study.

Creanor & co-workers (1986b) compared one week plaque isolated from natural tooth surface and from an enamel section mounted on an intra-oral appliance, in one subject. The tooth microflora was similar to the results of the present study, quoted above. The proportion of positive cocci isolated from enamel section plaque was higher than from natural tooth, and this is in agreement with the findings of the current study.

The enamel sections mounted on the intra-oral appliance, required for quantification of enamel demineralisation, have only a small unvarnished edge, which is adjacent to the acrylic of the appliance. Therefore, the plaque sampled from the section is in continuity with the plaque overlying the acrylic. Thus a similarity would be expected between the microflora of enamel section plaque compared with acrylic plaque. This was confirmed experimentally with the relative proportions of the four main groups of organisms being almost identical for section and acrylic plaques, while both were significantly different from natural tooth plaque with respect to positive cocci and bacilli.

On most occasions, slabs mounted on intra-oral appliances are covered with a Dacron gauze, to enhance the colonisation of the site (Ostrom et al., 1977; Gallagher & Pearce, 1979). Although this procedure encourages the proliferation of organisms on the slab, it reduces the similarity to the natural colonisation of tooth surfaces. In the present study, it was decided that an attempt would be made to reproduce the natural colonisation process, so the slabs were left uncovered. The slabs were mounted proud on the appliance, therefore the problem of proximity of enamel section to acrylic should not have arisen in this case. It might be expected, therefore, that the predominant cultivable flora in slab plaque would more closely mimic natural tooth plaque than did the plaque obtained from the enamel sections. The mean proportions of the main groups of organisms in the slab plaque were more similar to the counts obtained from tooth plaque than were the results from section and acrylic, but again, the difference between tooth and slab plaque was significant.

At the genus and species level, the mean percentage count of the individual streptococcal and Actinomyces spp. demonstrated a surface effect, with Strep. mutans being lower, and the others higher, in section, slab and acrylic plaques compared with tooth plaque. Among the less frequently isolated organisms, members of Fusobacterium and Capnocytophaga spp. were isolated from each surface on some occasions and no species was isolated from only one of the four surfaces on more than one occasion.

Thus, although there were quantitative differences in the predominant cultivable flora isolated from plaque obtained from the four surfaces, generally all results found in this study are within the ranges quoted for one week supragingival plaque microflora. No qualitative differences were found between the surfaces, and the variation in the results is consistent with other studies where considerable differences have been found in the microbial composition of plaque obtained from different tooth surface sites (Bowden et al., 1975).

pH Measurements and Acid Anion Profiles.

Plaque pH measurements, before and following sucrose exposure, were carried out on the material removed from the natural tooth surface and enamel slab at the end of the one week experimental period. This procedure could only be carried out on the three Glasgow subjects since there was no access to the facilities required for performing the pH measurements at Port Sunlight. However, samples for acid anion analysis were collected from all five subjects as such samples can be stored for short periods prior to analysis. In addition to the baseline collections, samples were removed for acid anion analysis six minutes after the application of the 10 % sucrose mouthrinse, since previous work has shown that both the plaque pH minimum, and the highest concentrations of total identifiable acid are produced about this time (Geddes, 1975).

The pH curves obtained for both tooth surface and slab plaques were similar, with no significant differences being found between them, and the minimum pH being recorded at either 3 or 6 minutes following sucrose application in the three subjects. As continuous monitoring of the pH

was not carried out, it is not possible to know when exactly the lowest value was obtained in each individual.

Acetate normally comprises the largest proportion of the acid anions found in fasting or resting plaque samples (Distler & Kroncke, 1983), but following exposure to sucrose the total amount of acid increases, with lactate increasing greatly from its resting level and becoming the dominant acid, while the concentration of acetate falls slightly (Geddes, 1984).

Although it was not possible to obtain concentrations of the plaque acids, as expressed per mg wet weight, because some of the samples were very small, the lactate / acetate ratios, before and following sucrose exposure, were calculated. The initial ratio was very similar to that found by Geddes (1975), with the altered ratio at six minutes reflecting the increased lactate, although the increase was not as marked as that of previous studies (Geddes, 1975; Distler & Kröncke, 1983). There was no significant difference between the lactate / acetate ratios of plaque from tooth surface or slab, for either baseline or six minute results, and these findings are in accordance with those of Creanor and co-workers (1986b) in their study on tooth surface and enamel section plaque.

No demineralisation was found on any of the enamel sections following the one week experimental period, and it was therefore decided that for the main demineralisation study, this should be extended to three weeks.

The use of thin enamel sections allows correlation between the microbial composition of plaque and mineral loss in the underlying enamel, while enamel slabs permit absolute counts of plaque microorganisms, which is particularly useful for early colonisation experiments. Therefore, in view of these attributes, and as no qualitative differences were found between the plaque removed from exogenously derived tooth surfaces and the natural tooth surface, with regard to microbial composition, pH and acid anion profiles, the results obtained suggest that the use of these enamel specimens is appropriate for the investigation of the early development of plaque flora and the microbial aetiology of early demineralisation.

3.4. Effect of Sucrose Concentration and Exposure Time on Plaque Microflora, pH and Acid Anions - an *in situ* Study.

3.4.1. Introduction.

As discussed in Chapter I, numerous studies have shown an association between the intake of fermentable carbohydrate and the incidence and severity of enamel demineralisation. Sucrose is metabolised by plaque bacteria to form acid end-products, and can be transformed by specific bacteria to form extra-cellular polysaccharides which increase plaque bulk, and may create a more acidic environment leading to a population shift which may change the cariogenic potential of the plaque (Minah *et al.*, 1985). The extra-cellular polysaccharides formed from sucrose may promote the accumulation of *Strep. mutans* in plaque, and it has also been suggested that the extra-cellular matrix material may enhance demineralisation by altering the diffusion properties of plaque (Zero *et al.*, 1986). This disaccharide is therefore often used in demineralisation experiments to increase the cariogenicity of the system.

In vitro studies have examined the effects of varying sucrose concentrations and different lengths of substrate exposure on pH changes in bacterial systems (Lagerlöf *et al.*, 1985). *In situ* demineralisation appliance models have used a range of different sugar concentrations as oral rinses (Tehrani *et al.*, 1983; Brudevold *et al.*, 1984) and for extra-oral application (Koulourides *et al.*, 1976; Ostrom & Koulourides, 1976) while some *in vivo* models have used mouthrinses containing up to 50 % w/v sucrose concentrations (von der Fehr *et al.*, 1970; Geddes *et al.*, 1978).

One of the aims of the appliance study described in Chapter IV was to develop a system which would be capable of producing rapid demineralisation and, in an attempt to increase the cariogenic potential of the immediate environment, sucrose solutions were applied extra-orally to some of the test sites. Thus this preliminary study was designed to compare i] the microbial composition of plaque, ii] the plaque pH response and acid anion profiles, and iii] the changes in mineral content of exposed enamel sites, following applications of either a 5 % or 10 % sucrose solution. The effect on plaque pH of various lengths of exposure time to extra-oral sucrose was also assessed, together with the plaque pH response

on both the left and right test areas of the appliance, when sucrose was added to only one side of the device. This study was carried out using one volunteer, on three separate three-week experimental runs.

3.4.2. Effect of Sucrose Concentration.

General Appliance Protocol.

The appliance was worn continuously by the volunteer for periods of three weeks, only being removed twice daily for toothbrushing using a non-fluoridated dentifrice (Unilever Research, Port Sunlight, England). The subject was also instructed to clean the appliance on these occasions using a toothbrush and water, but to leave the trough area untouched throughout the experimental period. The volunteer was permitted to rinse each side of the appliance under gently running tap water for a maximum of 3 seconds before replacing it in the mouth, and was advised to floss between the teeth once per day in all areas of the mouth, paying particular attention to the lower molar regions. The normal diet of the subject was maintained during each experimental period.

Experimental Method.

The appliance was worn for periods of three weeks as described above. The appliance was removed nine times daily and the trough area on one side received four drops (appoximately 200 µl) of a 5 % w/v sucrose solution whilst the other received the same volume of a 10 % sucrose solution. Sterile plastic dropper bottles (Fig 3.4) were used to dispense the solutions with 1.5 - 2 hourly intervals between applications. After three weeks, dental plaque was removed (as described previously in 2.5.1. and 2.5.2.) from the enamel windows and the surface of some of the slabs, in order to calculate the total counts and the percentage of individual groups of bacteria in the plaque sample, (see 2.5.5.). Baseline and post-experimental microradiographs were taken (see 2.4.1.) and changes in enamel mineral content were measured by microdensitometry (2.4.2.).

pH Response and Acid Anion Profiles.

This experiment was carried out to determine whether the concentration of

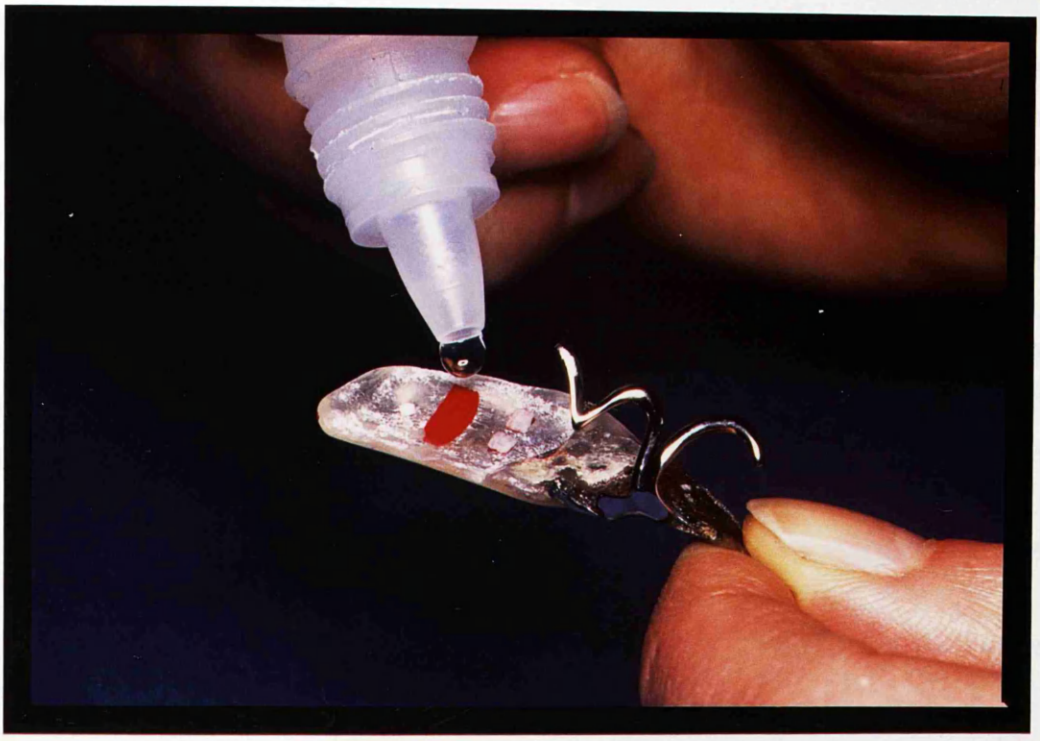


Fig. 3.4. **Application of 10 % sucrose solution to trough area of appliance, using plastic dropper bottle.**

sucrose applied extra-orally during the experimental period affected the pH response and acid anion profile of plaque, following short-term immersion in sucrose, at the end of the 3 week run, and also to establish whether varying the sucrose concentration of the solution used for the immersion affected these parameters.

Accordingly, at the end of the 3 week experimental period, and after the plaque samples had been removed for microbiological investigation, two overnight fasting plaque samples were also removed from unsampled slabs on each side of the appliance. The pH of one sample from each side was measured using the method described in 2.7.2., whilst the other sample was stored for subsequent acid anion analysis (see section 2.7.1.). The trough areas of the appliance were then inserted into separate glass beakers each containing a 5 % sucrose solution as shown in Fig 3.5. After 30 s the appliance was removed from the sucrose and replaced in the mouth. After a further 6 min, plaque samples were again removed from slabs on both sides of the appliance and all samples were then processed for isotachophoresis. The appliance was again replaced in the mouth, and the volunteer refrained from eating and drinking for a further 4 hours when the procedure was repeated, except that on this occasion, the baseline plaque was a resting sample and the trough areas were placed in 10 % sucrose solutions for 30 s. On each occasion the plaque pH responses following sucrose application were recorded as described in Section 2.7.2.

3.4.3. Effect of Sucrose Exposure Time.

The purpose of this study was to assess the effect of various sucrose exposure times on plaque pH measurement. An appliance, fitted with enamel slabs, was worn by one volunteer for periods of one week, during which time the normal diet was maintained and no additional stressing employed. After seven days, plaque samples were removed from the enamel slabs for pH measurement both before and after sucrose applications as described in Section 2.7.2. This procedure was carried out on three separate occasions. On each day of sampling, the trough areas of the appliance were inserted into separate glass beakers, each containing a 10 % w/v sucrose solution for periods of one, two and three minutes, with the appliance being returned to the mouth for at least three hours between each application, to allow the plaque to return to resting pH

Fig. 3.5. Trough areas of appliance inserted into separate glass beakers, each containing a sucrose solution.

levels in the interim. The sequence of sucrose applications was rotated, so that the order of the sucrose exposure durations was different on each occasion.

3.4.4. Effect of Unilateral Sucrose Application.

It was anticipated that the appliance model would allow the effect of various experimental conditions to be investigated at the same time on the different sides of the appliance. This study was designed to determine whether this assumption was valid, by ascertaining whether or not a sucrose solution could be applied to one side of the device without affecting the plaque pH on the opposite side.

The appliance was worn for one week as described in Section 3.4.3. and at the end of this period it was removed from the mouth and baseline plaque pH measurements recorded as described in 2.7.2. The trough area on one side then received four drops (approximately 200 µl) of a 10 % w/v sucrose solution (see 3.4.2.) whilst the other side remained untreated. After one minute the excess sucrose solution was allowed to drain from the trough area and the appliance was reinserted in the mouth. The pH of the plaque from each side of the appliance was then measured during the subsequent 30 min as described in Section 2.7.2. This study was performed on two occasions on one volunteer, with the sucrose being applied to the other side of the appliance on the second run.

3.4.5. Results.

Effect of Sucrose Concentration on Microbiological Flora.

The predominant cultivable plaque flora, expressed as mean percentage of total cfu, isolated from enamel sections and slabs, after three weeks' exposure to either 5 % or 10 % sucrose, are shown in Tables 3.19. and 3.20. The proportions of microorganisms for sections and slabs combined are given in Table 3.21. The statistical analysis of the results obtained in this experiment is shown in Table 3.22. Occasional differences were seen between the three experimental runs, the position on the section, and the location of section or slab on the appliance, and the side of the appliance from which the plaque was isolated within each treatment group.

However, there was no significant difference between the 5 % and 10 % sucrose groups for any of the isolates under study.

The microbial counts from the above experiment, expressed as $\log_{10}$ cfu/mm^2 enamel slab surface are shown in Table 3.23. The analysis of this appears in Table 3.24., and again there is no significant difference between microbial counts obtained using the two sucrose concentrations. Thus, no difference in either bacterial counts or relative proportions of bacteria isolated was found, when comparing the 5 % and 10 % sucrose applications.

Effect of Sucrose Concentration on Enamel Demineralisation.

The enamel sections used in the experiment were microradiographed before and following the three weeks' sucrose application, to assess the extent to which mineral loss had occurred. The parameters measured are shown in Table 3.25. The integrated mineral loss over the experimental period was expressed as the Δz value, this being the calculated value at three weeks minus the integrated mineral loss measurement at baseline. The Surface Zone (SZ) and Lesion Body (LB) values quoted are again the changes observed in these parameters over the experimental period (ie. three week value minus baseline). These parameters have been described in Section 2.4.2.

The results show that negligible mineral loss occurred, with no significant difference between the Δz and SZ values for the two treatments. Although the LB values were very small, the demineralisation resulting from 10 % sucrose is slightly larger, and this reaches significance at the 5 % level. However, as so little mineral loss occurred on any occasion, this is of doubtful relevance.

Effect of Sucrose Concentration on Plaque pH Measurement.

At the end of the three week experimental periods, during which one side of the appliance had received 5 % sucrose and the other 10 % sucrose, the plaque pH on both sides of the appliance was measured before and following the application of 5 % and 10 % sucrose - a period of four hours being left between the two applications to allow the plaque to return to

its resting pH. The results of the three experimental runs, and the mean values and standard deviations, are shown in Tables 3.27. and 3.28. The ΔpH measurements (baseline pH minus minimum pH) are given in Table 3.29. With both 5 % and 10 % sucrose application immediately prior to pH measurement, whether the plaque had been exposed to 5 % or 10 % sucrose during the three week growth period did not significantly affect the ΔpH or minimum pH values (paired t-test analysis).

Similarly, for the plaque exposed to 5 % or 10 % sucrose during the experimental period, whether the plaque received 5 % or 10 % sucrose immediately prior to pH measurement did not significantly affect the ΔpH values, although minimum pH was significantly lower following the single application of 10 % sucrose ($p < 0.01$).

Effect of Sucrose Concentration on Plaque Acid Anions.

In this experiment, the acid anion profile of the plaques was determined at baseline (ie following the three week growth period) and six minutes after application of 5 % or 10 % sucrose. The results are given in Tables 3.30. and 3.31. The mean lactate, acetate and total identifiable acid concentrations at baseline ranged from 1.96 - 4.40, 20.39 - 24.67, and 31.31 - 39.57 nmol/mg wet weight plaque respectively. Six minutes following sucrose application, the equivalent figures were 21.83 - 28.38, 13.13 - 18.17 and 46.56 - 57.70 nmol/mg wet weight plaque. Student's t-test analysis of the results showed that whether 5 % or 10 % sucrose was used during the three week growth period, and whether 5 % or 10 % sucrose was applied following baseline analysis, did not significantly affect the six minute production of lactate, acetate or total identifiable acid anions.

Effect of Sucrose Exposure Time on Plaque pH Measurement.

One week old plaque on enamel slabs was exposed to 10 % sucrose for periods of one, two and three minutes, as described in Section 3.4.3., and measurement of plaque pH was made prior to and following sucrose application. The results are shown in Fig 3.6, and it can be seen that for each exposure time, the mean values show a typical pH response to sucrose, with the range in minimum pH from 5.25 - 5.48. One way analysis of variance revealed no significant difference in the ΔpH or

minimum pH value, in the three groups representing the different exposure times.

Effect of Unilateral Sucrose Application.

pH measurements of plaque on both sides of the appliance were made following unilateral application of sucrose, as described in Section 3.4.4. The resulting plaque pH curves are shown in Fig 3.7. As this experiment was performed on only two occasions, statistical analysis has not been carried out, but an obvious difference in pH responses can be seen, with the plaque exposed to sucrose showing a typical Stephan curve, while the unexposed plaque had only a slight pH fall.

SECTION
5 %

	F[a]	Mean[b] (SD)[c]	Median	Range
+ve cocci	12/12	32.5 (14.5)	34.5	4.8 - 54.8
S. mutans	3/12	0.4 (1.2)	ND[d]	ND - 4.0
S. sanguis	7/12	12.1 (14.4)	4.2	ND - 37.5
S. oralis	9/12	13.4 (13.0)	7.9	ND - 36.4
-ve cocci	9/12	8.4 (10.4)	3.8	ND - 33.3
Veillonella	9/12	8.1 (10.4)	3.8	ND - 33.3
+ve bacilli	12/12	48.4 (20.5)	48.2	20.0 - 83.3
Actinomyces	12/12	41.0 (21.9)	41.6	7.7 - 78.5
Lactobacillus	5/12	4.0 (8.3)	ND	ND - 26.9
-ve bacilli	10/12	10.7 (8.4)	11.2	ND - 28.0
Bacteroides	8/12	8.3 (6.8)	9.4	ND - 18.2

SECTION
10 %

	F	Mean (SD)	Median	Range
+ve cocci	12/12	33.3 (17.9)	34.6	6.7 - 58.3
S. mutans	7/12	0.8 (2.7)	0.1	ND - 9.5
S. sanguis	10/12	13.1 (10.7)	10.2	ND - 30.0
S. oralis	12/12	14.8 (7.0)	15.6	4.0 - 25.0
-ve cocci	9/12	8.2 (9.1)	6.0	ND - 31.8
Veillonella	8/12	6.8 (9.0)	4.0	ND - 31.8
+ve bacilli	12/12	39.0 (27.8)	24.8	4.2 - 86.7
Actinomyces	11/12	32.9 (26.8)	22.8	ND - 76.0
Lactobacillus	8/12	0.8 (1.7)	0.1	ND - 5.0
-ve bacilli	11/12	19.4 (11.9)	18.6	ND - 36.4
Bacteroides	10/12	14.0 (10.1)	14.0	ND - 31.8

[a] - Isolation frequency ; [b] - Mean percentage of total cfu ;
[c] - Standard deviation ; [d] - Not detectable.

Table 3.19. % predominant cultivable microflora isolated from enamel section plaque after 3 weeks' 5 % and 10 % sucrose applications, n=12.

SLAB
5 %

	F	Mean (SD)	Median	Range
+ve cocci	12/12	29.8 (25.8)	18.6	0.1 - 73.1
S. mutans	5/12	0.5 (1.6)	ND	ND - 5.6
S. sanguis	5/12	6.6 (11.9)	ND	ND - 38.5
S. oralis	10/12	21.2 (22.3)	13.6	ND - 73.1
-ve cocci	6/12	3.3 (4.0)	1.8	ND - 10.3
Veillonella	6/12	3.0 (3.6)	1.8	ND - 10.3
+ve bacilli	12/12	52.0 (24.5)	61.4	7.7 - 75.0
Actinomyces	12/12	40.8 (24.4)	40.7	7.7 - 75.0
Lactobacillus	5/12	2.0 (4.4)	ND	ND - 14.8
-ve bacilli	12/12	14.9 (8.7)	13.4	3.6 - 31.0
Bacteroides	10/12	11.8 (10.3)	12.0	ND - 28.6

SLAB
10 %

	F	Mean (SD)	Median	Range
+ve cocci	11/12	22.6 (20.7)	18.6	ND - 56.2
S. mutans	8/12	0.4 (1.2)	0.1	ND - 4.3
S. sanguis	8/12	9.9 (11.9)	4.0	ND - 32.1
S. oralis	5/12	7.9 (10.3)	ND	ND - 24.1
-ve cocci	9/12	7.4 (8.0)	4.6	ND - 22.2
Veillonella	9/12	6.8 (6.9)	4.6	ND - 20.8
+ve bacilli	12/12	50.7 (25.3)	58.7	7.4 - 88.9
Actinomyces	12/12	43.8 (21.6)	46.2	7.4 - 77.8
Lactobacillus	6/12	1.6 (3.4)	0.05	ND - 11.1
-ve bacilli	12/12	19.2 (17.3)	13.0	3.7 - 59.2
Bacteroides	9/12	13.5 (15.2)	8.5	ND - 48.1

Table 3.20. % predominant cultivable microflora isolated from enamel slab plaque after 3 weeks' 5 % and 10 % sucrose applications, n=12.

SECTION + SLAB
5 %

	F	Mean (SD)	Median	Range
+ve cocci	24/24	31.1 (20.5)	30.0	0.1 - 73.1
S. mutans	8/24	0.4 (1.4)	ND	ND - 5.6
S. sanguis	12/24	9.4 (13.2)	1.7	ND - 38.5
S. oralis	19/24	17.3 (18.3)	11.4	ND - 73.1
-ve cocci	15/24	5.9 (8.2)	3.4	ND - 33.3
Veillonella	15/24	5.6 (8.0)	3.4	ND - 33.3
+ve bacilli	24/24	50.0 (22.2)	51.6	7.7 - 83.3
Actinomyces	24/24	40.9 (22.7)	40.7	7.7 - 78.5
Lactobacillus	10/24	3.0 (6.6)	ND	ND - 26.9
-ve bacilli	22/24	12.8 (8.6)	12.2	ND - 31.0
Bacteroides	18/24	10.0 (8.7)	9.8	ND - 28.6

SECTION + SLAB
10 %

	F	Mean (SD)	Median	Range
+ve cocci	23/24	28.0 (19.7)	27.4	ND - 58.3
S. mutans	15/24	0.6 (2.1)	0.1	ND - 9.5
S. sanguis	18/24	11.5 (11.2)	8.0	ND - 32.1
S. oralis	17/24	11.3 (9.3)	12.0	ND - 25.0
-ve cocci	18/24	7.8 (8.4)	4.6	ND - 31.8
Veillonella	17/24	6.8 (7.9)	4.2	ND - 31.8
+ve bacilli	24/24	44.8 (26.7)	40.4	4.2 - 88.9
Actinomyces	23/24	38.4 (24.4)	32.8	ND - 77.8
Lactobacillus	14/24	1.2 (2.6)	0.1	ND - 11.1
-ve bacilli	23/24	19.3 (14.5)	16.5	ND - 59.2
Bacteroides	19/24	13.7 (12.6)	10.0	ND - 48.1

Table 3.21. % predominant cultivable microflora isolated from enamel section and slab plaque after 3 weeks' 5 % and 10 % sucrose applications, n=24.

	Treatment	Position	Run	Side
+ve cocci	NS	**	NS	NS
S. mutans	NS	NS	NS	NS
S. sanguis	NS	NS	NS	NS
S. oralis	NS	*	NS	NS
-ve cocci	NS	NS	*	NS
Veillonella	NS	*	*	NS
+ve bacilli	NS	NS	*	*
Actinomyces	NS	NS	*	**
Lactobacillus	NS	NS	NS	NS
-ve bacilli	NS	NS	NS	NS
Bacteroides	NS	NS	NS	NS

NS = Not significant ; * = $p < 0.05$; ** = $p < 0.01$.

Table 3.22. Statistical analysis of effect of treatment, position, run and side on percentage microbial counts in 5 % and 10 % sucrose plaque, obtained from enamel sections and slabs.

	5 % sucrose		10 % sucrose	
	Mean	(SD)	Mean	(SD)
+ve cocci	7.17	(0.86)	7.02	(0.90)
S. mutans	1.44	(1.92)	1.71	(2.08)
S. sanguis	6.46	(0.91)	6.70	(0.75)
S. oralis	6.98	(0.88)	6.48	(1.04)
-ve cocci	6.44	(0.53)	6.67	(0.65)
Veillonella	6.42	(0.52)	6.66	(0.65)
+ve bacilli	7.51	(0.58)	7.52	(0.71)
Actinomyces	7.38	(0.58)	7.47	(0.68)
Lactobacillus	2.00	(2.80)	2.33	(2.60)
-ve bacilli	7.02	(0.61)	7.08	(0.65)
Bacteroides	6.83	(0.64)	6.74	(0.66)
Total	7.86	(0.56)	7.88	(0.62)

Table 3.23. Mean microbial counts ($\log_{10}$ cfu/mm^2 enamel slab surface) of enamel slab plaque after 3 weeks' 5 % and 10 % sucrose applications, n=12.

	Treatment	Position	Run	Side
+ve cocci	NS	NS	**	NS
S. mutans	NS	NS	*	NS
S. sanguis	NS	NS	*	NS
S. oralis	NS	NS	**	NS
-ve cocci	NS	NS	*	NS
Veillonella	NS	NS	*	NS
+ve bacilli	NS	NS	***	NS
Actinomyces	NS	NS	**	NS
Lactobacillus	NS	NS	NS	NS
-ve bacilli	NS	NS	*	NS
Bacteroides	NS	NS	NS	NS
Total	NS	NS	***	NS

NS = Not significant ; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Table 3.24. Statistical analysis of effect of treatment, position, run and side on $\log_{10}$ microbial counts in 5 % and 10 % sucrose plaque, obtained from enamel slabs.

5 % SUCROSE	Mean	(SE)	Median	Range
Δz^a	93.4	(27.5)	65.0	0.0 - 255.0
SZ^b	0.58	(0.21)	0.22	0.0 - 1.98
LB^c	0.59	(0.17)	0.22	0.0 - 1.98

10 % SUCROSE	Mean	(SE)	Median	Range
Δz	155.1	(40.5)	129.0	0.0 - 436.0
SZ	1.01	(0.38)	0.28	0.0 - 3.97
LB	1.59	(0.46)	1.08	0.0 - 4.70

[a] - Δz = total mineral loss (% vol min x μm) ; [b] - SZ = Surface Zone (% vol min) ; [c] - LB = Lesion Body (% vol min).

Table 3.25. Change in demineralisation parameters of enamel sections following 3 weeks' 5 % and 10 % sucrose applications, n=12.

	Treatment	Position	Run	Side
Δz	NS	NS	NS	NS
SZ	NS	NS	NS	NS
LB	*	NS	NS	NS

NS = Not significant ; * = $p < 0.05$.

Table 3.26. Statistical analysis of effect of treatment, position, run and side on enamel demineralisation parameters following 3 weeks' 5 % and 10 % sucrose applications.

5 % SUCROSE PLAQUE, 5 % SUCROSE IMMERSION

Time (min)	Experimental Run Number			
	1	2	3	Mean (SD)
0	6.88	7.43	7.08	7.13 (0.28)
1	6.53	6.32	6.61	6.49 (0.15)
3	5.97	5.55	6.12	5.88 (0.30)
6	5.88	5.17	5.86	5.64 (0.40)
10	5.70	5.68	6.76	6.05 (0.62)
15	6.87	6.15	7.40	6.81 (0.63)
20	6.78	6.21	7.74	6.91 (0.77)
30	6.75	6.68	7.45	6.96 (0.42)

5 % SUCROSE PLAQUE, 10 % SUCROSE IMMERSION

Time (min)	Experimental Run Number			
	1	2	3	Mean (SD)
0	6.71	7.09	7.29	7.03 (0.29)
1	6.92	5.06	6.31	6.10 (0.95)
3	6.09	4.86	5.59	5.51 (0.62)
6	5.97	4.88	4.97	5.27 (0.60)
10	5.14	4.99	5.64	5.26 (0.34)
15	4.39	6.19	6.74	6.11 (0.68)
20	6.36	6.37	6.71	6.48 (0.20)
30	6.52	6.62	7.23	6.79 (0.38)

Table 3.27. 5 % sucrose plaque pH response in each of three experimental runs, and mean pH response, following immersion in 5 % and 10 % sucrose for one minute.

10 % SUCROSE PLAQUE, 5 % SUCROSE IMMERSION

Time (min)	Experimental Run Number			
	1	2	3	Mean (SD)
0	7.65	7.38	7.32	7.45 (0.18)
1	5.79	5.82	5.93	5.85 (0.07)
3	5.50	5.32	5.54	5.45 (0.12)
6	5.92	5.74	5.70	5.79 (0.12)
10	5.56	6.13	6.71	6.13 (0.58)
15	5.70	6.43	7.13	6.42 (0.58)
20	6.24	6.49	6.89	6.54 (0.33)
30	7.60	6.64	7.08	7.11 (0.48)

10 % SUCROSE PLAQUE, 10 % SUCROSE IMMERSION

Time (min)	Experimental Run Number			
	1	2	3	Mean (SD)
0	7.14	7.00	7.57	7.24 (0.30)
1	6.15	5.17	5.31	5.54 (0.53)
3	5.39	4.72	4.94	5.02 (0.34)
6	5.14	4.83	5.10	5.02 (0.17)
10	5.68	5.26	6.03	5.66 (0.38)
15	5.84	6.12	6.71	6.22 (0.44)
20	6.09	6.39	6.30	6.26 (0.15)
30	6.71	6.64	6.90	6.75 (0.13)

Table 3.28. 10 % sucrose plaque pH response in each of three experimental runs, and mean pH response, following immersion in 5 % and 10 % sucrose for one minute.

5 % SUCROSE PLAQUE

	Experimental Run Number			
	1	2	3	Mean (SD)
5 % sucrose	1.18	2.26	1.22	1.55 (0.61)
10 % sucrose	1.57	2.23	2.32	2.04 (0.41)

10 % SUCROSE PLAQUE

	Experimental Run Number			
	1	2	3	Mean (SD)
5 % sucrose	2.15	2.06	1.78	2.00 (0.19)
10 % sucrose	2.00	2.28	2.63	2.30 (0.32)

Table 3.29. ΔpH of 5 % and 10 % sucrose plaques in each of three experimental runs, and mean ΔpH, following immersion in 5 % and 10 % sucrose for one minute.

5 % SUCROSE PLAQUE, 5 % SUCROSE IMMERSION

	Time (min) 0	6
	Mean (SD)	Mean (SD)
Formate	2.07 (1.01)	1.15 (1.19)
Pyruvate	2.95 (1.58)	2.93 (1.50)
Phosphate	9.43 (6.13)	6.21 (2.42)
Lactate	4.40 (2.65)	21.83 (13.78)
Succinate	1.87 (0.88)	1.00 (0.27)
Acetate	24.67 (7.82)	18.17 (5.67)
Propionate	3.59 (1.52)	2.56 (0.88)
Total	39.55 (14.78)	47.63 (19.84)

5 % SUCROSE PLAQUE, 10 % SUCROSE IMMERSION

	Time (min) 0	6
	Mean (SD)	Mean (SD)
Formate	0.69 (0.42)	0.81 (0.73)
Pyruvate	1.97 (0.88)	2.37 (0.87)
Phosphate	6.05 (3.35)	4.55 (1.64)
Lactate	2.52 (1.22)	25.41 (10.88)
Succinate	1.62 (0.74)	1.33 (0.47)
Acetate	21.55 (7.95)	16.30 (2.15)
Propionate	3.49 (1.91)	2.38 (0.88)
Total	31.83 (12.04)	48.61 (14.68)

Table 3.30. 5 % sucrose plaque acid anions (nmol/mg wet weight plaque) before and 6 minutes following immersion in 5 % and 10 % sucrose, for one minute, n=3.

10 % SUCROSE PLAQUE, 5 % SUCROSE IMMERSION

	Time (min) 0	6
	Mean (SD)	Mean (SD)
Formate	0.96 (1.09)	1.58 (0.86)
Pyruvate	2.88 (1.72)	3.46 (1.45)
Phosphate	9.94 (8.71)	8.24 (3.78)
Lactate	4.35 (3.49)	28.38 (7.94)
Succinate	2.10 (1.36)	1.25 (0.49)
Acetate	21.42 (9.74)	19.85 (2.37)
Propionate	4.43 (2.62)	3.18 (0.88)
Total	36.13 (19.04)	57.70 (9.88)

10 % SUCROSE PLAQUE, 10 % SUCROSE IMMERSION

	Time (min) 0	6
	Mean (SD)	Mean (SD)
Formate	1.06 (0.51)	0.43 (0.75)
Pyruvate	3.10 (1.48)	3.36 (1.51)
Phosphate	6.50 (3.64)	5.09 (2.86)
Lactate	1.96 (1.04)	26.52 (11.72)
Succinate	1.28 (0.64)	1.17 (0.08)
Acetate	20.39 (11.82)	13.13 (4.78)
Propionate	3.52 (2.19)	1.95 (0.64)
Total	31.31 (16.58)	46.56 (18.75)

Table 3.31. 10 % sucrose plaque acid anions (nmol/mg wet weight plaque) before and 6 minutes following immersion in 5 % and 10 % sucrose, for one minute, n=3.

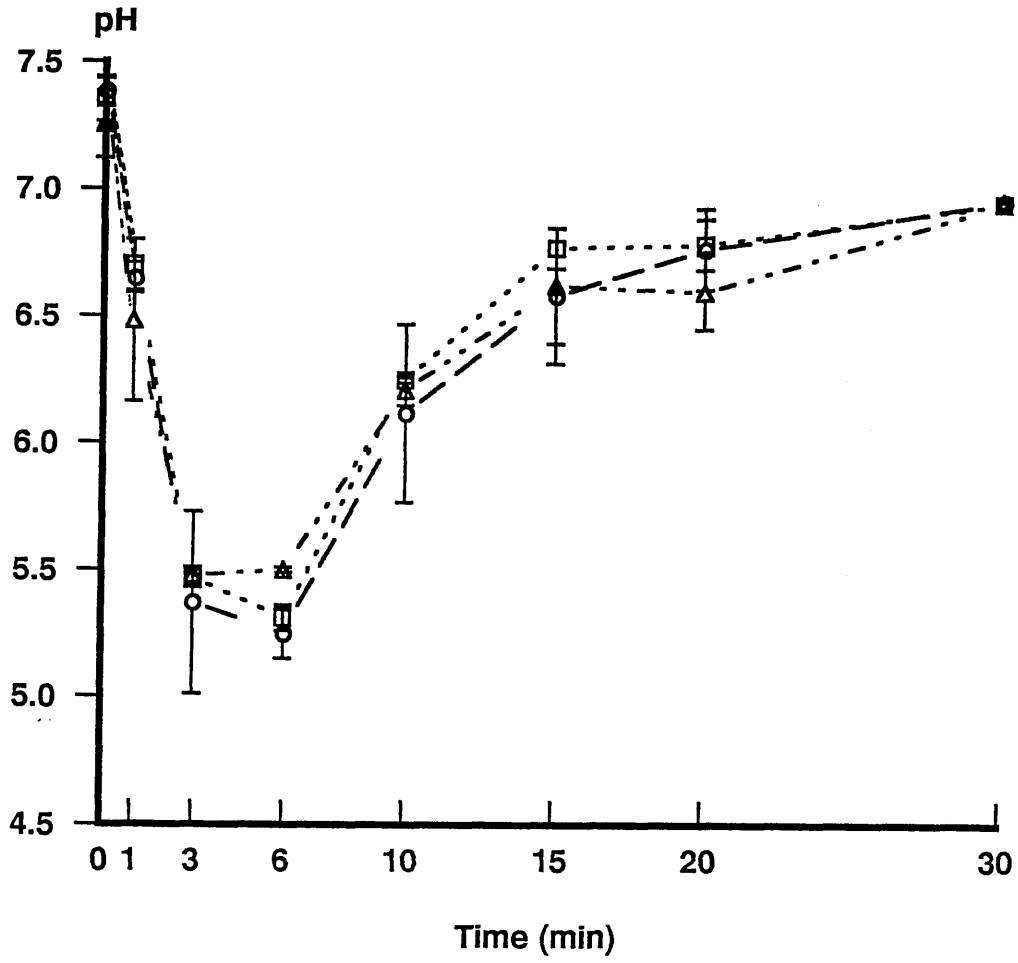


Fig. 3.6. Mean (SD) plaque pH response to one (square), two (circle) and three (triangle) minute exposure to a 10 % sucrose solution, n=3.

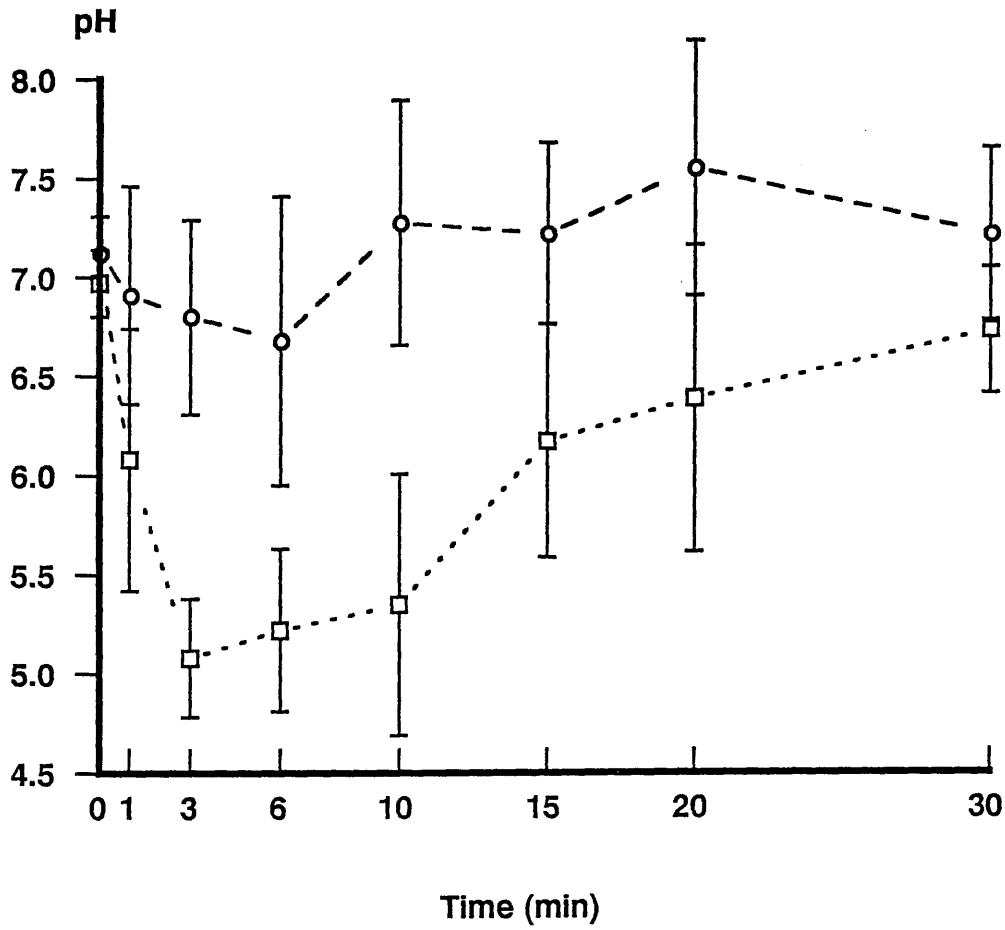


Fig. 3.7. **Mean (SD) plaque pH response to unilateral 10 % sucrose application, on exposed (square) and unexposed (circle) sides, n=2.**

3.4.6. Discussion.

Many studies have shown large differences in the bacterial composition of plaque obtained from different tooth sites and at different times of sampling. The very large variation obtained for percentages of cocci and bacilli in plaque samples has meant that it is very difficult to obtain a baseline for plaque bacteriology against which changes produced by diet can be measured (Bibby, 1976).

This difficulty was apparent in the present study since, although the use of one subject eliminated the problem of inter-subject variation and the sites sampled were in close proximity on each side of the appliance, significant differences in the proportion of some bacteria occurred with regard to position, side and run.

As a consequence of such problems, information available from other studies on the effect of diet on plaque composition is mainly limited to the differences in the percentage distribution of specific bacteria, such as streptococci and lactobacilli (Folke et al., 1972; Staat et al., 1975; Minah et al., 1985) and the bulk of plaque formed on the surface (Carlsson & Egelberg, 1965). In addition, the concentration, frequency and method of applying sucrose has varied in different studies, and therefore a comparison of bacterial flora obtained from the plaques exposed to 5 % and 10 % sucrose in this study, cannot be directly compared with other studies where the effect of sucrose on smooth surface plaques was investigated. The composition of the microfloras obtained in this study, from the plaque samples exposed to 5 % and 10 % sucrose solutions during the three-week growth period were, however, within the range of results obtained from other studies on mature smooth surface plaques (van Palenstein Helderman, 1981).

Preliminary studies involving the same individual had shown that direct extra-oral 10 % sucrose applications had caused no change in plaque microbial composition, and little increase in demineralisation of enamel sections, compared to unstressed plaque. It was considered that the lack of increase in mineral loss may have been due to the relatively high sucrose concentration inhibiting the plaque microflora. This study was therefore performed to assess whether frequent extra-oral applications of

5 % sucrose would produce a change in the microbial composition and an increase in demineralisation, compared to the effect of 10 % sucrose applications.

No significant difference in the composition of the predominant cultivable flora was found between the two sucrose concentrations, with regard to either the relative proportion of the total cultivable flora or counts per mm^2 enamel. This included the Strep. mutans and Lactobacillus spp. which comprised only a small fraction of the microflora under both treatment conditions. It therefore appears that when comparing the two concentrations of sucrose no particular bacterial species were selected by one concentration, and that other factors influencing the microenvironment surrounding the sampling sites and the normal background diet of the individual, may have been at least as important in determining the bacterial composition of the plaque samples.

As the plaque which accumulated during the three-week experimental periods was required for pH measurements and acid anion analysis, in addition to microbiological studies, sufficient material was not available for weighing, and therefore, whether or not either of the concentrations produced a greater bulk of plaque by the production of extra-cellular polysaccharides, is not known.

The amount of demineralisation, measured as the change in total mineral loss from the baseline, on the enamel sections following exposure to the sucrose solutions, was minimal. However, the changes that did occur showed a slight increase in the mean Δz, surface zone and lesion body values in sections which had received 10 % sucrose, with the difference between the two sucrose concentrations reaching significant levels for lesion body values. A study by Tehrani and co-workers (1986), measuring the changes in mineral content of enamel slabs mounted on an intra-oral appliance and exposed to 5 % and 10 % glucose mouthrinses for various lengths of time, found that the demineralisation, measured by change in iodide permeability, was higher with the 10 % glucose solution, particularly for short rinsing periods. Glucose was used in this study, since previous work had shown that it produces enamel demineralisation scores similar to that of sucrose (Brudevold et al., 1983), and the former sugar is easier to measure in studies on salivary clearance.

Lagerlöf and co-workers (1985) studied the effects of different concentrations of sucrose on pH changes produced by Strep. mitior in an artificial mouth. They found, in a system which simulates the process of oral sugar clearance, the pH fall was dependent on initial sucrose concentration up to 10 %, but was independent of higher concentrations.

The method used in the present study involved immersing the test sites of the appliance in the sucrose solution for periods of one minute, with the solution being kept at room temperature and the appliance being replaced in the mouth after the sucrose had been allowed to drain from the trough area. While this procedure is different to the in vivo rinsing and in vitro artificial mouth systems described above, the results of this study also found that the application of a 10 % sucrose solution to three-week plaque produced a larger fall in plaque pH than did a 5 % solution, although the plaques exposed to the different solutions over the three-week experimental periods did not show any significant differences in their response to the application of sucrose solutions.

The concentrations of acid anions present in the fasting and resting plaque samples were similar to those obtained from other studies (Geddes, 1975; Distler & Kröncke, 1983), the total identifiable acid consisting mainly of volatiles, with acetate being present in highest concentration. The plaques analysed from samples removed following application of both 5 % and 10 % sucrose solutions showed similar responses, with an increase in the total concentration of identified acid. Lactate showed the greatest increase, while the concentrations of acetate and propionate both decreased. These findings were again similar to other results (Geddes, 1975), and no significant differences were found between the data obtained for the different concentrations of sucrose. The results of the plaque response to various durations of 10 % sucrose exposure showed that the minimum pH and the maximum change in pH from resting levels occurred following two minutes' application of sucrose. Lagerlöf and co-workers (1985) found that exposure of bacteria, in an artificial mouth system, to sucrose for a period of two minutes gave an almost maximum pH fall compared to the pH fall resulting from much longer exposures. No significant differences were found in the present study between the one, two and three minute applications, as the plaque receiving the one

minute application produced almost the same pH response as the plaque exposed for longer periods of time.

As the 10 % sucrose produced a greater fall in plaque pH, and slightly more demineralisation, compared to the 5 % solution, and did not appear to be inhibitory towards any of the plaque flora, it was decided to use 10 % sucrose in the main demineralisation study.

Frequency of sucrose application has often been shown to be important regarding the initiation of demineralisation (Rugg-Gunn, 1983). Consequently sucrose solutions were applied nine times daily, both in this preliminary investigation and in the subsequent study. This frequency of sucrose application was used in the in vivo studies of von der Fehr and co-workers (1970) and Geddes and co-workers (1978).

Since it was important that the subjects followed the experimental protocols, attempts were made in the main study to make the frequent sucrose applications as easy as possible for volunteers by giving them dropper bottles which were kept at room temperature. The bottles were used to dispense the sucrose, which was applied for only one minute, since the preliminary results had shown that this produced a change in pH similar to that obtained from longer applications.

The results obtained from the study on unilateral sucrose exposure showed that only a small change in plaque pH occurred on the opposite side of the appliance, and it was therefore considered satisfactory to compare the effects of sucrose and no stressing on the plaque flora and changes in mineral content of enamel, at the same time on opposite sides of the appliance.

3.5. Variation in Enamel Susceptibility to Demineralisation - An *in vitro* Study.

3.5.1. Introduction.

One of the difficulties of attempting to investigate the composition of the plaque flora associated with enamel demineralisation is the variation in the susceptibility of the tissue to cariogenic challenge. In addition to permitting the assessment of mineral content to be made both pre- and post-experimentally, the use of thin enamel sections in this study allowed multiple specimens to be prepared from a single tooth for use both on opposite sides of an appliance within a subject and in different subjects as described in Section 2.3.2. However, investigations have shown that differences in caries susceptibility occur even on different sites on the same tooth (de Groot *et al.*, 1986) and this study was therefore designed to compare the variation in susceptibility which occurred between i] sections obtained from different teeth, ii] sections obtained from the same tooth, and iii] different sites on each enamel section, using a standard *in vitro* demineralising system.

In addition, for sections obtained from two teeth, one of the sections was abraded on the outer enamel surface, while the other section was not, in an attempt to determine whether an increased amount of demineralisation, and a more uniform pattern of mineral loss, occurred on abraded sections.

3.5.2. Methods.

Two of the enamel sections prepared from each tooth and used to provide sections for the *in situ* demineralisation study, described in Chapter IV, were employed in this experiment. The sections which were to be left intact, were cut, hand-ground to a final thickness of 100-120 μm, and radiographed as described in Sections 2.3. and 2.4.1. For the abraded sections, the half of the tooth from which the sections were to be obtained, was abraded to a depth of approximately 100 μm, prior to sectioning. The sections obtained were then ground and radiographed as above. Further details of the abrading procedure will be given in Section 4.4.2. The cut surfaces, of both abraded and non-abraded sections were

then covered with two coats of nail varnish (see 2.3.5.), but on this occasion the outer enamel edge was left completely unvarnished.

Each section was then placed in a separate plastic bijou bottle containing 5 ml of a demineralising solution (see Appendix III). The solution was changed daily, and after one week, the sections were removed from the bottles and re-radiographed. The image of the enamel on each section was divided into five equal portions from the occlusal to the cervical margin and microdensitometric assessment of changes in mineral content (see 2.4.2.) in each of these five sites was made. A comparison of the amount of demineralisation was made both between one section and another and also among different areas of the same section.

3.5.3. Results.

Susceptibility of Enamel to Demineralisation.

The mean Δz of the five positions on each section, and for both sections from each tooth combined, are shown in Table 3.32. Analysis of variance showed no significant difference in the mean Δz of the eight teeth, and no significant difference between the mean Δz of the two sections from each tooth. However, it can be seen from the values of the standard error of the mean that there were large differences in the amount of demineralisation produced on the five sites on each section. The mean Δz values for each site on each tooth are given in Table 3.33. Results are shown for only six of the teeth, as in the other two, the surface of one section was abraded and the other non-abraded, and therefore combining the results from the two sections would not be appropriate. On two occasions, the amount of demineralisation at position five was so great that the enamel surface was destroyed, and quantification of mineral loss was not possible. These two sites were therefore excluded from statistical analysis which still, however, revealed higher levels of demineralisation in position five (ie. the cervical region of the section), compared to all other sites on the sections ($p < 0.05$).

A summary of the results for each position, for all non-abraded sections combined, is given in Table 3.34., where the higher level of demineralisation in position five can be seen more clearly.

For the two abraded enamel sections, the mean Δz values for each position are shown in Table 3.35. Although there was at least as much variation in mineral loss at different positions and between the two sections, the amount of demineralisation was greater than that which occurred in non-abraded sections ($p < 0.05$).

Tooth	Section 1 (SE)[a]	Section 2 (SE)	Sections 1 + 2 (SE)
1	3608 (1640)	3125 (598)	3367 (731)
2	4194 (971)	2888 (961)	3541 (670)
3	2879 (753)	1347 (394)	2113 (470)
4	1283 (470)	1652 (467)	1447 (312)
5	2348 (807)	3256 (662)	2802 (508)
6	2360 (866)	2968 (537)	2630 (495)
7	3171 (957)	4198* (417)	3685 (492)
8	1378 (603)	3534* (1219)	2456 (726)

[a] = Standard error of the mean ; * = Abraded enamel sections.

Table 3.32. Mean Δz (% vol min x μm) of enamel sections obtained from eight teeth, following <u>in vitro</u> demineralisation for one week, n=5.

NORMAL ENAMEL

Tooth	Position 1 (SE)	2 (SE)	3 (SE)	4 (SE)	5 (SE)
1	1892 (1452)	4262 (489)	3872 (2748)	1586 (1261)	5220 (1667)
2	3226 (991)	3532 (1260)	864 (843)	4470 (2128)	5614 (34)
3	1695 (237)	846 (472)	3631 (1216)	1194 (616)	3202 (1301)
4	640 (524)	1381 (864)	1328 (892)	1656 (376)	3009 -*
5	2554 (739)	880 (275)	3951 (1233)	3170 (1864)	3447 (632)
6	4172 (401)	2020 (2025)	2706 (114)	1146 (323)	3585 -*

* = Unmeasureable due to destruction of enamel surface.

Table 3.33. Mean Δz (% vol min x μm) of enamel section positions obtained from six non-abraded teeth, following <u>in vitro</u> demineralisation for one week, n=2.

NORMAL ENAMEL

Position	Mean (SE)	Median	Range
1	2058 (416)	1874	118 - 4572
2	2011 (451)	1572	0 - 4788
3	2672 (515)	2505	24 - 6611
4	2443 (513)	1919	329 - 6592
5*	4092 (404)	3831	1905 - 6882

* = 2 sites unmeasureable due to destruction of enamel surface.

Table 3.34. Overall Δz (% vol min x μm) of enamel section positions from non-abraded teeth, following one week in vitro demineralisation, n=12.

ABRADED ENAMEL

Position	Mean (SE)	Median	Range
1	2687 (1037)	2687	1653 - 3721
2	5382 (1992)	5382	3395 - 7368
3	2803 (1043)	2803	1763 - 3843
4	2912 (1391)	2912	1525 - 4298
5	5549 (186)	5549	5363 - 5735

Table 3.35. Overall Δz (% vol min x μm) of enamel section positions from abraded teeth, following one week in vitro demineralisation, n=2.

3.5.4. Discussion.

It is recognised that human teeth vary in susceptibility to the development of demineralisation (Nikiforuk, 1985), and that even different parts of the same tooth have varying predispositions to carious attack (de Groot et al., 1986). The results of this study generally agree with these findings, with the extent of demineralisation varying as much at different sites on the same tooth, as between teeth. The reason for the varying susceptibility is not known, but one possibility is thought to be the inhomogeneity of the enamel fluoride content (Weatherell et al., 1977). Fluoride is incorporated into the surface enamel during localised episodes of de- and remineralisation and then has an inhibitory influence on further caries development (Larsen & Fejerskov, 1977).

In this study, significantly greater demineralisation occurred at the cervical regions of the teeth than at any other area of the enamel sections. However, Creanor and co-workers (1988) found the opposite, with more mineral loss at the incisal area. One possible explanation for the results of the latter study is that on buccal surfaces more de- and remineralisation episodes occur adjacent to the gingival margin where plaque accumulation is greatest. Hence the fluoride thereby precipitated may have had a protective effect on enamel during the subsequent in vitro study. However, in both studies, the teeth used had been extracted for orthodontic purposes and therefore had not been erupted long enough for many episodes of de- and remineralisation to have occurred. It is conceivable that as teeth erupt this process could occur at successive levels on the tooth surface, as each forms the gingival margin for a period of time. In a newly fully erupted tooth, therefore, there may not have been sufficient time for the cervical margin to have experienced episodes of de- and remineralisation, thus no protective effect would be seen at this site. This is a possible explanation for the findings of the present study, where more mineral loss occurred at the cervical region of the tooth, while other areas experienced a similar, lesser, amount of demineralisation. Other possible explanations for varying enamel susceptibility in different areas of a tooth are the varying orientation of crystalites and prisms within the enamel, affecting the permeability of the enamel surface (Shellis, 1984), and the fact that the composition of enamel

is not homogeneous, with cervical enamel having a lower mineral density than occlusal enamel (Poole et al., 1981).

de Groot and co-workers (1986) found that abrading the enamel surface resulted in lesions that were both larger and more homogeneous compared to those found on non-abraded sections, as abrasion removed the surface layer containing irregular amounts of fluoride. While the current study showed that abrasion of the enamel surface was associated with greater amounts of demineralisation, large variations in mineral loss were still seen. This may reflect the fact that abrasion of only 100 μm was made in this study, while de Groot and co-workers abraded their sections to a maximum depth of 500 μm. Although, overall, more demineralisation occurred in abraded sections, considerable variation in susceptibility was still seen, and therefore non-abraded sections were used in the main in situ experiment described in Chapter IV.

It has to be recognised that an in vitro study cannot reproduce accurately the conditions of the oral environment. Therefore, although results show the variability in the susceptibility of enamel to demineralisation, care must be taken when extrapolating to the in vivo situation.

As it has been demonstrated that there is as much variation in enamel susceptibility within the same tooth as between teeth, there is no advantage in ensuring that all sections used in an experiment are derived from the same tooth. It has to be accepted that this variability exists and is unavoidable if one is attempting to investigate the relationship between plaque microflora and the initiation of enamel demineralisation, and this has to be borne in mind when interpreting the results. In the main study, in order to reduce the variability in susceptibility of different regions on the tooth , the extremities of the sections were excluded, and the "windows" were sited so as to correspond with positions two and four as described in this in vitro study.

3.6. Conclusions.

From the results of the preliminary experiments described in this Chapter, it was decided that the following techniques and methodology would be used in subsequent investigations:-

i] On the in situ appliance, enamel sections would be used for correlation of plaque microflora and demineralisation, while enamel slabs would be used for assessment of absolute microbial counts.

ii] Enamel test sites would be located around the central positions of the enamel sections, in an attempt to reduce the known variability of the sites with regard to susceptibility to demineralisation.

iii] Sucrose, in a concentration of 10 %, would be used in an attempt to promote enamel demineralisation.

iv] Sonication of the plaque samples would be performed, following vortex mixing, to achieve more uniform distribution of bacterial cells within the suspension, prior to dilution and plating.

v] The plaque microflora would be determined by identification of all colonies from a single region, containing 30-50 colonies, on a single blood agar plate.

CHAPTER IV.

IN SITU DEMINERALISATION STUDIES.

4.1 Introduction.

As discussed in Section 1.12., two theories exist regarding the microbial aetiology of dental caries - the Specific and Non-Specific Plaque Hypotheses. The former suggests that a small number of bacterial species play a major role in the initiation of caries, whilst the latter suggests that a wide range of the acidogenic plaque microflora may be capable of producing lesions. Human cross-sectional and longitudinal studies have been employed in an attempt to resolve this issue. However, whilst many cross-sectional studies have shown an association between Strep. mutans and caries (Duchin & van Houte, 1978; Meiers et al., 1982; Carlsson et al., 1985), they have been unable to prove a cause and effect relationship (reviewed by Edwardsson, 1986). Although longitudinal studies are more appropriate for demonstrating such a relationship they also produce results which are difficult to interpret, because of various problems related to methodology eg i] the lengthy course of the disease process, ii] difficulties in early detection of lesions, iii] the large number of sites that have to be monitored and iv] the difficulties in accurate plaque sampling from carious sites. Thus the problem remains unresolved.

One method of overcoming some of these problems is to use an appliance model, and the introduction of the intra-oral cariogenicity test (Koulourides et al., 1976), involving the use of enamel slabs mounted on an intra-oral appliance, enabled rapid demineralisation to be produced in the oral environment. Most studies employing this model have investigated the cariogenicity of different sugars (Brudevold et al., 1984; Tehrani et al., 1986). Only a few have identified the plaque flora associated with demineralisation (Ostrom et al., 1977; Gallagher & Pearce, 1979), and in these studies the enamel surface has been covered with gauze, in an attempt to promote plaque accumulation. As a result, it was not possible to relate discrete areas of enamel demineralisation to their overlying plaque. In addition, Ostrom and co-workers (1977) found that plaque which developed on gauze was of different composition to that forming on natural enamel surfaces.

The use of exogenously derived enamel specimens in intra-oral demineralisation tests overcomes the ethical difficulties associated with attempting to create carious lesions in the oral environment. Various techniques have been employed to assess the mineral loss that has occurred in these specimens, including microradiography and microdensitometry (Bergman & Lind, 1966); polarised light microscopy (Kidd, 1983) and microhardness testing (Koulourides et al., 1974). Other methods currently in use include assessment of iodide permeability (Brudevold et al., 1984) and scanning electron microscopy, (Clarkson et al., 1984; Ingram & Fejerskov, 1986).

The large number of techniques available for mineral content assessment thus makes interpretation and comparison of results from different studies difficult.

Since variation in the porosity, fluoride content and chemical composition of the enamel surface layer may cause irregularities in carious lesion formation (Brudevold et al., 1982; Pearce, 1983) and the removal of the enamel surface may increase caries susceptibility (Theuns et al., 1983), many in vitro and in situ studies have used abraded enamel in their demineralisation investigations.

The main aim of this study was to overcome some of the difficulties mentioned above, by the use of an appliance model which would allow rapid demineralisation to occur, and plaque to develop on enamel sections, without a gauze covering being required. Thus the relationship between microbial plaque composition and mineral content of discrete enamel sites could be assessed. The effect of extra-oral sucrose applications, implantation of Strep. mutans and abrasion of the enamel section surface pre-experimentally on the plaque microflora, in relation to demineralisation, was also investigated. These in situ studies were performed using the methods developed as described in Chapter III.

The main demineralisation study was carried out, employing both normal dietary conditions, and experimental protocols likely to increase the cariogenic potential of the environment surrounding the enamel test site.

4.2. Subject Characteristics.

4.2.1. Introduction.

The intra-oral appliances were worn by seven volunteers, three from Glasgow Dental Hospital and four from Unilever Research Laboratories, Port Sunlight. Ethical approval was obtained from Greater Glasgow Health Board and Unilever Research, and the volunteers each received written details of their role in the project then signed a consent form agreeing to participate in the study (see Appendix IV).

4.2.2. Tests for Caries Risk.

Initially, some commonly used caries-predictive screening tests were performed, to ensure that a range of caries-risk status existed among the volunteers. The following tests were carried out on each subject.

Clinical Examination.

The current and previous caries experience was measured by recording the decayed, missing and filled surfaces of the teeth. The individuals were examined in a dental chair using an overhead light, a mirror and a William's probe. Bite-wing radiographs were not taken as the Ethics Committee did not consider them to be a necessary part of the study.

Salivary Characteristics.

Five minute, mixed, stimulated, salivary samples were collected on three separate occasions from each volunteer. The subject chewed on a sterile 1.0 g piece of paraffin wax and expectorated, over a five minute period, mixed saliva into a sterile plastic graduated universal container. The quantity of saliva collected was measured against the scale on the outside of the container and the flow rate per minute calculated. A 1.5 ml sample was then removed from the universal container, using a sterile plastic syringe and needle, and 1.0 ml injected into a glass ampoule containing Dentobuff solution (Dentobuff, Orion Diagnostica, Espoo, Finland). The ampoule was then shaken and left at room temperature for three to five minutes as directed by the manufacturer, and the colour

change of the solution was compared with a colour chart to determine the buffering capacity of the salivary sample.

Ten-fold dilutions of the remainder of the sample were made in ABB, and using a spiral plater, 50 µl of the 10^{-1}, 10^{-2} and 10^{-3} dilutions were inoculated on to both MSB and Rogosa SL agars as described in Section 2.5.4. The plates were incubated at 37°C in 5 % CO_2 in air for three days, and were then stamped with a grid which divided the surface into zones representing known volumes of inoculum. Two zones from equivalent positions on opposite sides of a plate were selected and all colonies present within these areas were counted. Three colonies from each zone were subcultured for identification. In this way the salivary counts of Strep. mutans and Lactobacillus spp. were determined from the MSB and Rogosa plates respectively, and were expressed as the number of cfu per ml of saliva.

Assessment of Plaque Acidogenic Potential.

The volunteers were asked to refrain from brushing the lingual surface of the lower molar teeth for one week, and at the end of this period, pooled plaque samples were collected from these unbrushed sites using a sterile dental excavator (No. 243, Ash, England). The plaque was used for analysis of acid anion content before and after a 10 % w/v sucrose mouthrinse as described in Section 2.7.1., and the results expressed as the plaque lactate/acetate ratio before, and six minutes after, the sucrose mouthrinse. This investigation was carried out on six of the seven volunteers.

4.3. Three Week In Situ Studies.

4.3.1. Experimental Protocols.

Enamel sections and slabs were prepared and mounted on to the appliance trough areas as described in Section 2.3. (Fig 2.7.).

The appliance was worn by each volunteer for periods of three weeks, during which time normal diet was maintained. The subjects were instructed to brush and floss their natural teeth and to clean the

appliance, as described in Section 3.4.2. At the start of each experiment, they were given a detailed protocol sheet which they were asked to follow for that particular run.

During each three-week period, two of three possible experimental protocols were followed, a different one being employed on each side of the appliance. The three experimental protocols were:

i] the appliance was worn as described above with no additional stressing.

ii] the appliance was removed nine times daily, at 1.5 to 2 hourly intervals during the waking period, and 4 drops (approximately 200 μl) of a sterile 10 % w/v sucrose solution was applied to the trough area on each occasion. After one minute the fluid was allowed to drain from the appliance which was then replaced in the mouth without rinsing. A sterile plastic dropper bottle was used to store and dispense the sucrose solution. Two bottles containing the solution were given to each of the volunteers at the beginning of each run, and they were asked to keep the one in use at room temperature. The other bottle was stored at 4°C for 10 days then used at room temperature for the remainder of the experimental run. Samples of the sucrose solutions were periodically cultured on blood agar plates throughout the three week periods, and on each occasion were found to be free from bacterial contamination.

iii] the trough area was inoculated with the volunteer's own Strep. mutans at 0, 4, 24, and 28 hours (vide infra). In addition, 10 % sucrose applications were employed as described in Protocol ii.

The study was designed so that three experimental runs would be carried out by each subject, resulting in eight enamel sites for each protocol, ie a total of 24 sites for each subject. However, two of the subjects withdrew during the study (see 4.5.1.).

At the end of the three-week experiment, the appliance was removed and plaque samples collected from the enamel windows of the sections (Section 2.5.1.) and the entire surface of the enamel slabs (2.5.2.), then immediately diluted (2.5.3.) and inoculated on to plates for culture as

described in Sections 2.5.4. and 2.5.5. The slabs were then removed with a sterile dental excavator before a solution of amyl acetate (BDH Chemicals Ltd., Poole, England) was applied to the trough area to allow the sections to be eased gently off the base with a small paint brush. The sections were then radiographed as described previously (Section 2.4.1.).

Microdensitometric analysis of the radiographs was then performed and the demineralisation data obtained for each enamel site was expressed in two forms, namely by Δz and lesion profile group, as described in Section 2.4.2.

4.3.2. Preparation of Strep. mutans and Inoculation on to Enamel Specimens.

A modification of the technique used by Brudevold and co-workers (1984) was employed to prepare Strep. mutans cells for inoculation on to enamel surfaces in an attempt to improve colonisation by this organism. An ampoule containing freeze-dried cells of Strep. mutans, previously isolated from the volunteer's own mouth, was opened and the contents re-constituted in 1 ml of ABB, as described in Section 2.6. The suspension was inoculated on to two blood agar plates and incubated anaerobically at 37°C for 24 hours. The identity of the organism was then checked using the API 20 Strep System as described in Section 2.5.5. and colonies were subcultured and incubated for a further 24 hours. The colonies were then removed from two of the blood agar plates using a sterile swab and cultured in 100 ml of Todd-Hewitt Broth (Gibco-Europe, Paisley, Scotland) (Appendix II), containing 2 % sucrose, for 18 hours at 37°C under anaerobic conditions, in a static culture. The resulting culture was centrifuged (20,000 g) and the pellet washed in 40 ml of a sterile solution of 50 mM NaCl and 20 mM $NaHCO_3$. Paraffin-stimulated whole saliva was obtained from each subject and clarified by centrifugation at 4°C for 10 min at 20,000 g (MSE High Speed 18 Centrifuge, MSE Ltd., Crawley, Sussex, England). The Strep. mutans cells from each volunteer were then suspended in 2 ml of the subject's own clarified saliva. Mixing of saliva and cells was carried out by sonication for 15 s at a setting of 1.5 immediately after the saliva had been prepared, and 200 µl of the resulting suspension was applied to the trough area of the appliance for 5 min.

After removing the excess fluid, the Strep. mutans suspension on the appliance was allowed to stabilise for one min. Before placing the appliance in the mouth, a sample of the residual matter on one enamel slab was removed by scraping with a sterile dental excavator (No 243, Ash, England) and dispersed by sonication and vortex mixing in 1 ml ABB. Dilutions of this were inoculated on to blood agar plates and incubated in 5 % CO_2 in air to check that viable cells had been inoculated on to the test site of the appliance. The entire procedure of inoculation and sampling was repeated at 4, 24 and 28 hours after the start of the experiment.

The remaining fluid from each prepared suspension which had not been inoculated on to the test sites was diluted in ABB and 50 µl of the 10^{-4} to 10^{-6} dilutions were inoculated on to blood agar plates using a spiral plater as described in Section 2.5.4. The plates were incubated for 3 days in 5 % CO_2 in air and viable counts determined using the counting grid, as previously described in Section 2.5.5. In this way, the concentration of cells inoculated on to the trough area of the appliance on each occasion was determined.

4.3.3. Dietary Assessment.

A diet diary recording the intake of each volunteer was completed during the second week of each three-week experiment. The subject was given a notepad and was asked to record each time an item was consumed, together with estimates of the quantity ingested and the length of time taken to consume it. At the end of each experimental period, each volunteer was interviewed about the way in which the diary had been completed, and the diaries were analysed with reference to McCance and Widdowson's "The Composition of Food" (Paul and Southgate, 1978). Using the results of food cariogenicity studies, the items consumed were then categorised, as far as possible, into foods with low and high cariogenic potential, beverages with cariogenic potential, and items with a possible protective potential such as fluoride-containing tea. The frequency of intake of the items in each of these categories was then determined for each subject for each day of the seven-day diet history in each experimental run.

4.4. Abraded - v - Sound Enamel - An In Situ Study.

4.4.1. Introduction.

The three-week appliance study was repeated on one volunteer, using both abraded and normal enamel, to assess whether the removal of the outer surface layer of the enamel affected either the amount of de-mineralisation produced, or the compostion of plaque developing on the surface.

4.4.2. Preparation of Enamel.

The crowns of caries-free premolars were sectioned as described previously in Section 2.3.2. The lingual portion was then mounted on an acrylic block for sectioning whilst the buccal surface was abraded by rotating the tooth by hand in a slurry of aluminium oxide powder on a ground glass plate. Measurements of the thickness of the buccal portion were made at six points along the surface using a micrometer, and approximately 100 μm of the enamel surface was removed, before mounting on to an acrylic block. Both halves of the tooth were then sectioned using a Leitz 1600 rotating annular diamond blade (see Section 2.3.2.), and hand-ground to a final thickness of between 100 and 120 μm, as previously described (Section 2.3.3.).

The sections were then varnished on all surfaces, leaving three unvarnished windows of approximately 1.5 mm on the natural enamel edge. One section with an abraded surface and one with an intact surface were then mounted on to each trough area as described in Section 2.3.6.

Eight enamel slabs were also prepared as described in Section 2.3.7. Approximately 100 μm of the surface enamel was then removed from four, by handgrinding in the aluminium oxide slurry on a ground glass plate, with measurements of enamel loss being taken intermittently using the micrometer, until the required reduction was attained. The surface area of each of the eight slabs was then measured by computerised planimetry as described in 2.3.8. (Fig. 2.8.), and two abraded and two non-abraded slabs were mounted on to each side of the appliance.

4.4.3. Experimental Protocols.

The appliance was worn by a single volunteer for periods of 3 weeks as described in Section 3.4.2. During the experimental period no additional stressing was employed on one side of the appliance, whilst the volunteer's own Strep. mutans was implanted on to the trough area of the other side, and one minute extra-oral 10 % sucrose applications were carried out nine times daily as described in Section 4.3.1. A total of three experimental runs were performed. After each three week period, the microflora of plaque overlying the enamel windows and slabs was identified (see 2.5.5.), and the sections microradiographed as described in 2.4.1.

4.5. Results.

4.5.1. Subject Characteristics.

Seven adult volunteers, age range 25 - 59 years, took part in this study. One subject completed only one three week experimental run, due to serious illness in the family, whilst another withdrew after two of the three runs due to pressure of work. The other five subjects completed all three experimental runs.

The salivary Strep. mutans and Lactobacillus spp. counts expressed as $\log_{10}$ counts per ml of saliva are shown in Table 4.1., together with the DMFS score of each subject. The mean salivary levels of Strep. mutans and Lactobacillus spp. ranged from 3.58 and 3.24 respectively in Subject A, to 5.92 and 5.79 in Subject E. Linear regression analysis showed a positive correlation ($p < 0.001$) between the Strep. mutans and Lactobacillus spp. counts for each subject. Subject E had the highest salivary counts of both organisms and the highest DMFS score, and although there was a trend towards an association between the subjects' past and present caries experience, and the salivary counts of these bacteria, this did not reach significance level.

The mixed stimulated salivary flow rate and salivary buffering capacity data (using the Dentobuff method) are given in Table 4.2. The lowest flow rates of 0.8 ml/min were obtained from Subjects A and E, while the other values ranged from 1.8 in Subject B to 3.5 ml/min in Subject D.

Subjects A and E also had the poorest salivary buffering capacity, in the "poor - intermediate" range according to the Dentobuff criteria, whilst the buffering capacities of the other subjects were in the "normal - good" range.

The lactate / acetate ratio of one week plaque samples, also given in Table 4.2., showed an increase in all six subjects at six minutes following a 10 % sucrose rinse, compared with resting values. This varied from an increase in proportion of lactate to acetate by a factor of 3.6 in Subject D, to an almost nine-fold increase in Subject A.

4.5.2. Sample Size in Appliance Study.

A total of 144 samples was removed from the enamel sections and 138 samples were obtained from the enamel slabs during this study, with over 11,000 isolates being identified.

Two subjects did not complete the three experimental runs and neither participated in the protocols which involved the inoculation of Strep. mutans. The number of samples obtained from the enamel sections under normal plaque conditions (NP), ie no additional stressing employed, sucrose conditions (SP) and sucrose conditions with the inoculation of Strep. mutans (SPM) were 52, 52 and 40 respectively, with the corresponding numbers for the slabs being 49, 50 and 39.

4.5.3. Proportion of Plaque Bacteria Remaining on Enamel Slabs Following Sampling.

Approximately 10 % of the enamel slabs were removed from the appliance, using a dental excavator immediately after plaque sampling had been carried out, and the surface of each of these slabs was then pressed into the surface of a blood agar plate as described in Section 2.5.2. On some occasions, no growth occurred following anaerobic culture, while on other occasions a few colonies were found. However, none of the bacterial counts which were obtained from this procedure accounted for more than 0.01 % of the total cultivable flora retrieved from scraping the plaque from the enamel surface.

4.5.4. Strep. mutans Inoculum Size.

The concentration of the suspension of Strep. mutans cells in clarified saliva which was used to inoculate enamel test sites on the appliance ranged from mean values of 9.3×10^7/ml saliva, in Subject A, to 3.0×10^8/ml, in Subject C. The counts obtained from enamel slabs following inoculation, and immediately prior to placing the appliance in the mouth, varied from 8.9×10^4 cfu per mm^2 enamel surface, in Subject D, to 2.3×10^5, in Subject C.

4.5.5. Effect of Side of Appliance on Plaque Microflora.

For each subject, with the exception of Subject G who only completed one experimental run, the treatment conditions applied to the plaque were balanced on each side of the appliance.

The predominant cultivable flora isolated from the enamel sections and slabs, on each side, under all treatment conditions combined, are shown in Tables 4.3. and 4.4. respectively. The mean, median and range of the proportion of the total anaerobic cultivable flora, for the Gram positive and negative cocci and bacilli, showed little difference between the two sides, and analyses of variance (shown in Tables 4.17. and 4.18.) found no significant side effect for any of these groups of organisms. At genus and species level, the values obtained from the different sides were again similar for both sections and slabs, with only one organism, Strep. sanguis showing a significant difference ($p < 0.05$) in mean percentage count between the two sides for the enamel slabs (7.6 % on left, 12.0 % on right).

4.5.6. Effect of Position of Enamel Site Within Test Area.

Each trough area of the appliance contained a mesial and distal section, as shown in Fig. 2.7., and two windows - superior and inferior - were positioned on each section. The mean proportional microbial composition of the plaque samples, obtained from enamel sections at each position within the trough area, are shown in Table 4.5. The mean percentage distribution of positive cocci ranged from 36.2 %, on the distal superior sites, to 43.6 % on both mesial superior and mesial inferior positions.

The mean proportions of negative cocci, positive bacilli and negative bacilli ranged from 7.8 - 10.2 %, 40.1 - 49.2 % and 6.0 - 6.8 % respectively among the different sites.

Four identically named positions also existed for enamel slabs mounted on the trough of an appliance with, on this occasion, each position corresponding to a separate slab. The position of the slabs on the appliance trough area is also shown in Fig. 2.7. The microbial composition isolated from the slabs is shown in Table 4.6., and again little variation was seen amongst the four positions, even at the genus and species level.

Analyses of variance (see Tables 4.17. and 4.18.) found that position had no significant effect on the microbial flora for either sections or slabs.

4.5.7. Effect of Subject on Enamel Section Plaque Microflora, for Each Treatment Condition.

The mean predominant cultivable plaque microflora isolated from enamel sections, under each of the three treatment conditions in each subject, is shown in Tables 4.7. to 4.9.

Normal Plaque Conditions.

Table 4.7. gives the percentage distribution of the predominant isolates obtained from plaque subjected to no additional stressing during the three-week experiments (NP). The positive cocci and bacilli, combined, dominated the microflora in all subjects, with the former predominating in two subjects (C and G), where they comprised 54.7 and 49.2 % of the total respectively, while the positive bacilli were isolated in higher proportion in all other subjects (mean range, 37.5 - 66.5 %). The negative cocci showed a large variation in percentage distribution, with a mean proportion of only 2.6 % in Subject D, but comprising 25.6 % of the flora in Subject G. The mean proportion of negative bacilli in the plaque samples was under 10 % in six of the subjects, whilst it accounted for a mean of 15.8 % in Subject F.

Differences in the composition of the plaque flora from the seven subjects were also apparent at genus and species level. Strep. mutans was not isolated from any of the samples from Subject G, but was found in every plaque in Subject E, where its mean proportion was 21.2 %. Lactobacillus spp. were isolated from every individual on at least three occasions, with a mean range from 0.04 % in Subject B to 8.9 % in Subject F. Veillonella spp. comprised the total, or almost the total amount of Gram negative cocci in all subjects, except Subject E, where Neisseria spp. predominated.

Sucrose Plaque Conditions.

As shown in Table 4.8., Gram positive cocci and bacilli combined, again dominated the organisms isolated from plaque which had been exposed to extra-oral sucrose applications nine times daily (SP), with positive cocci predominating in three subjects, and positive bacilli being present in highest proportions in the other four individuals. Gram negative cocci showed a narrower range of mean proportions among subjects (4.1 - 12.6 %) than was found in the NP samples, while the percentages of negative bacilli in SP, ranged from being undetectable in any sample in Subject G, to comprising a mean proportion of 12.6 % of the flora in Subject A.

Strep. mutans was again not detected in plaque obtained from Subject G, while it ranged from 0.1 to 11.9 % of the total in the others. Lactobacillus spp. were isolated from at least two plaque samples from each subject, and ranged from a mean proportion of 0.02 % in Subject B to 7.8 % in Subject G.

Sucrose Plaque with the Inoculation of Strep. mutans.

Plaque samples which accumulated with sucrose supplementation and pre-inoculation of Strep. mutans, were obtained from only five subjects, and the results of the mean microbial composition are shown in Table 4.9. Gram positive cocci predominated in plaque from three of the individuals, but in the other two subjects comprised only 15.2 and 24.3 % of the total cultivable flora. Gram positive bacilli were present in highest proportion from these two individuals' samples (59.6 % and 68.5 % of the total). The proportion of Gram negative cocci ranged from 3.5 % in Subject E to

14.2 % in Subject C, while negative bacilli ranged from 0.5 % in Subject E to 5.9 % in Subject B.

The isolation frequency of Strep. mutans was 4/8 in Subject B, 7/8 in Subject C and 8/8 in the other three individuals, with the mean proportions ranging from 0.05 % in Subject B to 55.3 % in Subject A. Lactobacillus spp. were isolated from at least six of the eight samples from each individual, and the mean proportions ranged from 1.5 % in Subject B to 21.5 % in Subject E.

The large standard deviations of the means, and the wide ranges of proportions found for the various organisms in each subject under the different treatment conditions, shows that large intra-subject variation occurred within plaque treatment groups. Despite this, analysis of variance showed that the differences amongst the subjects reached significant levels for all species and the four main groups except negative cocci (Table 4.17.).

4.5.8. Effect of Treatment Condition on Enamel Section Plaque Microflora for all Subjects Combined.

The predominant cultivable microflora obtained from plaque samples under each treatment condition for the five subjects who participated in all three protocols, is shown in Table 4.10.

The mean proportions of Gram positive cocci and bacilli among the three treatment groups were similar, ranging from 39.3 - 42.9 % and 42.7 - 46.2 % respectively. Gram negative cocci also showed little treatment effect, ranging from 8.4 % in the SP group to 9.0 % in the NP group. Negative bacilli accounted for less than 10 % of the cultivable flora in each treatment group, ranging from 3.4 % in SPM to 8.2 % in SP.

Analysis of variance (Table 4.17.) showed no treatment effect regarding Gram positive cocci and bacilli, and negative cocci, whilst a significant effect ($p < 0.05$) was found with regard to negative bacilli, the proportion isolated from sucrose plaque being significantly higher than from SPM.

At genus and species level, the treatment conditions had a highly significant effect on Strep. mutans proportions ($p < 0.001$), with SPM plaque containing a significantly higher mean percentage of this organism than the other two plaques - 21.0 % compared to 4.4 % (NP) and 2.8 % (SP). No significant difference was found between the proportional counts in the latter two treatment groups. The isolation frequency of Strep. mutans increased from 52 % (NP) to 70 % (SP) and 88 % (SPM). The difference between NP and SPM was highly significant ($p < 0.001$, using the χ^2 test).

SPM plaque contained significantly higher levels of Lactobacillus spp. ($p < 0.05$), with the mean proportions in the different treatment groups being 1.5 % (NP), 1.4 % (SP), and 7.7 % (SPM). The isolation frequency was similar in NP and SP (68 and 65 % respectively) but higher in SPM plaque at 88 %. The difference between SPM and both NP and SP was significant ($p < 0.05$, χ^2 test). Strep. sanguis and Strep. oralis levels were higher in the normal and sucrose plaques compared to the plaque inoculated with Strep. mutans, with the treatment effect being significant, ($p < 0.01$), with regard to Strep. oralis. None of the other major organisms analysed, including Veillonella and Actinomyces spp., showed any treatment effect.

The total microflora isolated from the enamel section plaque, under each of the three treatment conditions, for all subjects combined,is given in Table 4.11. Of the streptococci not detailed previously, Strep. salivarius was isolated in the greatest number, the mean percentage counts being 1.9 % (NP), 6.3 % (SP) and 0.6 % (SPM). Anaerobic streptococci were isolated in 13/52 samples (NP), 9/52 (SP) and 8/40 (SPM), and comprised between 1 and 2 % of the total flora in all three groups. Neisseria spp. accounted for only a small proportion of the negative cocci, having a mean percentage count of 1.3 % in the normal plaque samples, and less than 0.5 % in the other two treatment groups.

Of the Actinomyces spp., A. odontolyticus was isolated most frequently and in the highest proportion in all three groups, while the catalase positive and negative subdivisions of A. viscosus / naeslundii accounted for similar proportions in each group.

Capnocytophaga was isolated in plaque from 17/52 sections, in both normal and sucrose conditions, but in only 7/40 of samples in the SPM group.

4.5.9. Effect of Subject on Proportional Enamel Slab Plaque Microflora, for Each Treatment Condition.

The percentage distribution of the predominant isolates obtained from enamel slab plaque under the three experimental conditions for each subject, is shown in Tables 4.12. to 4.14. A total of six slabs was lost during the three-week experiments, with the losses occurring in Subjects A, B, C and D. Thus the total number of slabs for each treatment condition varied slightly from subject to subject.

Normal Plaque Conditions.

The percentage microbial counts obtained under normal plaque conditions are shown in Table 4.12. Under these conditions, the ranges amongst subjects in mean proportion of the total cultivable flora for Gram positive cocci and bacilli were 22.0 - 74.3 % and 8.8 - 71.9 % respectively, with the highest coccal and lowest bacillary mean count being found in Subject G. The corresponding ranges for Gram negative cocci and bacilli amongst the subjects were 2.5 - 14.1 % and 1.8 - 19.6 % respectively.

Strep. mutans was not detected in any plaque samples obtained from Subjects A and G, while this organism's mean percentage count from Subject E was 13.9 %.

The mean proportion of Lactobacillus spp. varied from 0.1 % in Subjects C and G, to 4.7 % in Subject F.

Sucrose Plaque Conditions.

As shown in Table 4.13., positive cocci accounted for 76.4 % of the total anaerobic cultivable flora in Subject G, and ranged from 16.6 to 50.2 % in the other six subjects. Correspondingly, Subject G had the lowest mean count of positive bacilli (2.0 %) while this group of organisms comprised between 33.4 and 56.8 % in the other subjects. The proportion of negative cocci was under 10 % in each subject, except Subject G, who

again did not fit into the general pattern, having a count of 20.9 %. In Subject F, the mean percentage of negative bacilli was 25.0, and in the other subjects, 0.7 to 14.0 %.

As previously, Strep. mutans was not isolated in any plaque sample from Subject G, and had the highest mean percentage count in Subject E (20.6 %). In each of the other subjects, Strep. mutans was isolated from at least two samples, and the mean count was 0.1 % or less. Lactobacillus spp. were isolated also in at least two samples from all subjects, ranging from a mean of 0.03 % (Subject B) to 2.2 % (Subject E).

Sucrose Plaque with Inoculation of Strep. mutans.

Table 4.14. shows the results for the predominant flora isolated from SPM plaque. The range of mean percentage counts for positive cocci was 13.9 - 64.3%, while positive bacilli varied from 26.0 % in Subject A to 72.1 % in Subject C. Negative cocci were less variable, accounting for 5.0 - 11.4 % of the total flora, in the five subjects, and negative bacilli ranged from 2.1 % in Subject E to 10.9 % in Subject D. Strep. mutans was isolated from at least six of the plaque samples from each subject, and ranged from 1.0 % in Subject B to 52.3 % in Subject E. Lactobacillus spp. were also present in at least six of the sites from each subject, accounting for 1.3 - 9.0 % of the total cultivable microflora.

Statistical analysis of variance shown in Table 4.18. confirms that for all groups, genera and species, with the exception of Lactobacillus spp., a significant subject effect occurred.

4.5.10. Effect of Treatment Conditions on Proportional Enamel Slab Plaque Microflora, for Five Subjects Combined.

The predominant flora isolated from plaque under each treatment condition for subjects A to E combined is shown in Table 4.15., and the statistical analysis of variance is given in Table 4.18. The isolation frequency of Strep. mutans increased from 62 % (NP), to 63 % (SP) and 90 % (SPM), with the difference between SPM and both NP and SP being significant at the 1 % level (χ^2 test). The medians of the percentage counts were 0.1, in both NP and SP plaque, and 4.3 % in plaque which had experienced SPM

conditions. Similarly, the mean proportion of Strep. mutans was highest under SPM conditions (21.2 %), with the values for NP and SP being 3.3 and 4.4 % respectively. Statistical analysis showed this difference to be highly significant ($p < 0.001$).

The isolation frequencies of Lactobacillus spp. obtained from the three treatment conditions were 70 % (NP), 63 % (SP) and 85 % (SPM), with the difference between SPM and SP being significant at the 5 % level (χ^2 test). The medians for each treatment condition was 0.1 %. As with the isolation frequencies, the mean was slightly lower in SP (0.9 %) and NP (1.0 %) than in SPM plaque (3.4 %), but this difference did not reach a significant level.

For negative bacilli, the isolation frequency, mean and median were all higher in SP plaque, compared to plaque grown under NP and SPM conditions, the differences in isolation frequency and mean being significant at the 1 % level. (I.F. - χ^2 test ; mean - analysis of variance).

No significant difference in the isolation frequency or means for Veillonella and Actinomyces spp. was seen when comparing NP, SP and SPM.

A complete listing of the organisms isolated from the enamel slabs, in each treatment group for all subjects combined, is given in Table 4.16.

Strep. salivarius was, as with enamel section plaque, the most abundant streptococcus, other than those already tabulated, with its mean percentage count ranging from 0.7 % (SPM) to 4.9 % (SP). Neisseria spp. accounted for less than 1 % of the total flora in all three treatment groups.

Of the Actinomyces spp., A. odontolyticus, and catalase positive and negative divisions of A. viscosus / naeslundii, were each present in similar proportions, with the catalase positive division being slightly higher in each treatment group.

4.5.11. Effect of Subject and Treatment Condition on Absolute Microbial Counts of Plaque Obtained from Enamel Slabs.

Effect of Subject on Microbial Counts.

The microbial counts for the predominant plaque microflora, and total microbial count, expressed as $\log_{10}$ cfu per mm^2 enamel surface, for each subject, under each treatment condition is shown in Table 4.19. As some of the predominant organisms were not detected on every plate used for identification of the flora, the minimum detection value for each sample was calculated after consideration of the dilution factors and volume of inoculum on the sampled area of the plate, together with the surface area of the enamel slab from which the plaque sample had been removed. Half the minimum detection value was then added to the count for each organism, including those where the organism was not detected (see Section 2.8). All the resulting values were then log transformed.

For normal plaque, the log of the total microbial count ranged from 6.63 in Subject A to 8.24 in Subject F. The range in sucrose plaque was 6.38 - 8.09, with the lowest value again being seen in Subject A and the highest in Subject F. For plaque samples which had received sucrose applications and inoculation of Strep. mutans, the lowest total count was 6.66 (Subject D) and the highest, 7.62 (Subject B).

Analysis of variance of these results is given in Table 4.21. A significant subject effect was found for the total microbial counts ($p < 0.001$), and the individual counts for organisms and groups ($p < 0.01$ for positive cocci and Strep. sanguis ; $p < 0.001$ for all others).

Effect of Treatment Condition on Microbial Counts.

To examine any possible effect of the different treatment conditions, the mean microbial counts under each treatment condition, for Subjects A - E combined were tabulated (Table 4.20.). Subjects F and G were excluded as their results were incomplete. The mean total microbial count for the five subjects was 7.18 (NP), 6.96 (SP) and 7.15 (SPM). Analysis of variance (Table 4.21.) showed that a significant treatment effect was seen with positive and negative bacilli, Strep. sanguis and Strep. oralis (all, $p <$

0.01); and also with Strep. mutans and Actinomyces spp. (both, $p < 0.001$), with the highest count for Strep. mutans being seen in SPM, and the highest Actinomyces spp. count occurring in NP. However, treatment conditions had no significant effect on the mean total microbial counts.

4.5.12. Effect of Treatment Conditions on Enamel Demineralisation.

The demineralisation parameters Δz, SZ and LB, for each subject under each treatment condition are given in Tables 4.22. - 4.24.

Minimal demineralisation was observed in sections from every subject under normal plaque conditions, with the mean Δz values ranging from 175, in Subject B, to 388 in Subject E, and the mean total, for all subjects combined, being 275 units. For sucrose plaque, slightly higher values were found, the mean Δz values being from 131 (Subject C) to 540 (Subject D) and the mean total Δz, 337 units. In enamel sections which had received sucrose plaque conditions with inoculation of Strep. mutans, higher levels of demineralisation were observed in all subjects, the Δz ranging from 256 (Subject C) to 917 (Subject E). The mean Δz for all subjects was 601 % vol min x μm. In all three treatment conditions, the change in surface zone and lesion body values were small, although the highest values were again seen for Subject E, under SPM conditions.

The demineralisation parameters for each subject, for all conditions combined, are shown in Table 4.25. The lowest amount of mineral loss occurred with Subject C and the highest with Subject E. No samples were available for Subjects F and G under SPM conditions, so the values for these subjects in this Table are not comparable.

The statistical analysis of the results obtained from this experiment is given in Table 4.26. The order of the treatment runs and the position of the enamel windows on the sections did not significantly affect the amount of demineralisation. The side of the appliance on which the enamel sections were sited did not affect the Δz or SZ, but significantly affected the LB values at the 5 % level.

Despite the wide variation in Δz between subjects, there was considerable

intra-subject variation, shown by the large standard errors, so there was no subject effect in the amount of demineralisation observed.

Analysis of variance showed that there was a significant treatment effect with demineralisation of enamel under SPM conditions being significantly greater than that associated with natural and sucrose plaques ($p < 0.01$ for Δz values, $p < 0.05$ for lesion body values). While the demineralisation levels were slightly higher in SP than NP, this did not reach a significant level.

4.5.13. Lesion Profile and Δz Groups.

The number of enamel sites in each lesion profile group for each subject is given in Table 4.27. In five subjects, at least 50 % of the sites showed no change in lesion profile from baseline (ie Profile Group 1), while for Subjects D and E, the percentages of sites in this profile group were 46 and 33 % respectively. In most subjects, there was evidence, of surface softening (Groups 2 and 3) and sub-surface demineralisation (Group 4) in some sites, the exception being Subject G who produced at most, a small degree of surface softening (ie Profile Group 2). Only eight sites, however, were available from this subject, and these, together with those from Subject F, had only been exposed to normal and sucrose treatment conditions.

The distribution of the enamel sites into Δz groups following the three-week experimental period is shown in Table 4.28. In four of the five subjects who completed all three experimental protocols at least 40 % of the test sites showed only minimal change in mineral content (Group 1), while in Subject E, 12.% fitted into this category. With the exception of Subjects B and G, at least one of the sites from each individual produced a change in Δz value of over 1000 % vol. min. x μm (Group 4). This occurred most frequently in Subjects D and E where 17 and 21 % of the areas fitted into this demineralisation group.

The distribution of the enamel sites in the lesion profile groups under each treatment condition is shown in Table 4.29. Under normal plaque conditions, 64 % of the enamel test sites showed no apparent post-experimental change in lesion profile, 27 % showed a small amount of

surface softening, with the percentage of sites showing a larger degree of surface softening and subsurface demineralisation being 4 and 6 % respectively. More of the sites obtained following sucrose plaque conditions fitted into profile Group 3 (14 %) and into profile Group 4 (10 %) compared with NP exposure, whilst the percentage distribution of sites within the Profile Groups 1 - 4 following SPM conditions was 32, 25, 15 and 27 % respectively.

Table 4.30. shows the number of enamel sites in each Δz group resulting from each treatment condition. Of the NP sites, 19 % had demineralisation of between 500 and 1000 units, and no sites had demineralisation of over 1,000 units. However, 23 % of the sites exposed to sucrose had mineral loss over 500 units, including 8 % with a value of over 1000. In the SPM group, 42.5 % of sites were distributed between Groups 3 & 4 with 22.5 % having mineral loss of over 1000 % vol. min.x μm. For all treatment groups combined, the distribution of the 144 enamel sites into the Δz groups was as follows - 62 in Group 1, 43 in Group 2, 26 in Group 3 and 13 in Group 4.

4.5.14. Relationship Between Plaque Microflora and Total Mineral Loss, Under Each Treatment Condition.

The mean and standard deviation for the predominant cultivable microflora associated with each Δz Group, for each treatment condition, is shown in Table 4.31.

For normal plaque, the mean proportion of Strep. mutans was 1.8 % (Δz Group 1), 5.4 % (Group 2) and 3.9 % (Group 3), while the corresponding figures for Lactobacillus spp. were 1.8 %, 5.2 % and 0.1 %. However, there were large differences between the counts in different samples, within each Δz Group, as can be seen from the large standard deviations quoted. An increase in isolation frequency (see Table 4.32.) for Strep. mutans was found, increasing from 24 % in Group 1 to 60 % in Group 3 - χ^2 testing showed a significant difference between Groups 2 and 3, and Group 1 ($p < 0.05$). The isolation frequency of lactobacilli was similar in all three groups. The mean proportions of Veillonella spp. decreased from 10.8 % in Group 1, to 8.5 % in Group 2 and 3.2 % in Group 3. The

frequency of detection of Veillonella and Bacteroides spp. decreased as mineral loss increased.

In sucrose plaque, no trend was seen for the mean proportion of Strep. mutans or Lactobacillus spp. in relation to the extent of demineralisation (Table 4.31.). However, the isolation frequency of Strep. mutans was higher in Groups 3 and 4, than in Groups 1 and 2 (Table 4.32.). This was not statistically significant, but when NP and SP results were combined, a significantly higher isolation frequency for Groups 3 and 4, compared with Group 1 was seen ($p < 0.05$, χ^2 test). Lactobacillus spp. results showed no pattern in relation to demineralisation.

Veillonella spp., under SP conditions, was highest in the group representing the greatest mineral loss. Strep. sanguis in general increased with increasing demineralisation, forming 8.4 % of the total flora in Group 1, and 20.0 % of the flora in Group 4, and the isolation frequency was slightly higher in Groups 3 and 4 than in the other Groups. However, the mean proportion of Strep. oralis was higher in Δz Groups 1 and 2, compared with Groups 3 and 4, although the isolation frequency was highest in Group 4. Bacteroides spp. isolation increased from Group 1 to Group 3, but was isolated in no sample where mineral loss exceeded 1,000 units.

Under SPM conditions, the proportion of Strep. mutans was higher in all Δz Groups, compared to all the results obtained for NP and SP (Table 4.31.). A marked rise in percentage count occurred, in the SPM groups, ranging from 8.9 % in Group 1 to 30.5 % in Group 4. The isolation frequency was more than 80 % for all Δz Groups, and rose to 100 % where demineralisation exceeded 1,000 units. The mean proportion of lactobacilli was considerably higher in Δz Group 4 than in all of the other three groups, where it comprised a similar fraction of the total flora. Although the isolation frequency was high in all groups, Lactobacillus spp. was not isolated from one of the samples associated with the highest demineralisation group. The mean proportions and isolation frequencies of Strep. sanguis and Strep. oralis decreased with increasing mineral loss. The mean proportion of Veillonella spp.decreased from Δz Group 1 to Group 4, but no trend was seen in relation to isolation frequency.

Actinomyces spp. showed no pattern throughout the demineralisation groups.

A summary of the median, mean and range of the proportions of Strep. mutans and Lactobacillus spp. in each Δz Group is shown in Tables 4.33. and 4.34. The large ranges indicate the extent of variation in proportions within each group. Therefore the medians are a better representation of the results than the means. The median count of Strep. mutans was either ND or 0.1 for Groups 1 to 3 under each treatment condition, but was 1.8 % in Group 4 for SP, and 33.3 % for Group 4 in SPM. Similarly, the median of the proportions of lactobacilli was 0.1 for all Groups 1 and 2, and Group 3 in the NP samples, but rose to 1.6 and 2.8 % respectively for Group 3 in SP and SPM. The median for Group 4 in the SP plaques fell again to 0.1, but was considerably higher in the SPM Group 4, at 23.9 %.

4.5.15. Plaque Microflora and Lesion Profiles.

The predominant cultivable plaque microflora isolated from each lesion profile group, for all subjects and all treatment conditions combined, is shown in Table 4.35. The mean proportion of Strep. mutans increased from 5.0 % in profile Group 1 to 14.0 % in Group 4, the group containing those enamel sites exhibiting subsurface demineralisation. The isolation frequencies increased correspondingly from 52 to 90 %. Strep. oralis decreased between Groups 1 and 4, and the statistical analysis of these results, shown in Table 4.38., confirmed that this was significant at the 5 % level. Strep. sanguis and Veillonella spp. varied little amongst the four lesion profile groups. Actinomyces spp. tended to increase in the higher profile groups, while Lactobacillus spp. was similar in Groups 1 and 2, but rose markedly in plaque associated with obvious surface softening and subsurface demineralisation. The medians of Strep. mutans and lactobacilli were highest in Group 3.

4.5.16. Relationship of Plaque Microflora to Total Mineral Loss under All Treatment Conditions Combined.

The percentage distribution of the predominant cultivable flora isolated from the different Δz groups in each subject is shown in Table 4.36.

In Subject A, the mean proportions of Strep. mutans isolated from Δz Groups 1 - 4, were 21.1, 13.4, 5.0 and 52.4 % repectively, with the isolation frequency increasing from 40 % in Group 1, to 75 % in Groups 2 and 3, and 100 % in Group 4. The median values increased from ND in Group 1, to 0.1 in Groups 2 and 3, and 52.4 % in Group 4. The mean percentage counts of Lactobacillus spp.were less than 1 % in Δz Groups 1 and 2, but increased to 4.1 % and 6.3 % in Groups 3 and 4. The proportion of Veillonella spp. isolated from sites with mineral loss of under 500 % vol min x μm was 8.8 %, while lower levels of 2.0 - 4.0 % were associated with higher amounts of demineralisation. The mean Actinomyces spp. levels ranged from 16.9 % in Δz Group 2 to 39.3 % in Group 3. The proportions of Strep. oralis and Strep. sanguis in Group 4 were less than in the other three groups.

The mean proportions of Strep. mutans isolated from plaque samples obtained from each demineralisation group in Subject B were low, ranging from 0.01 % in Group 2 to 1.5 % in Group 1. No enamel sites experienced mineral loss in excess of 1,000 units and, with the exception of Actinomyces spp. which showed a slight increase in proportion from Δz Group 1 to Group 3, no obvious pattern was observed regarding the relationship between the percentage distribution of the organisms and levels of demineralisation.

In Subject C, mineral loss of over 500 Δz units occurred at only two sites, with minimal demineralisation occurring at most sites (17/24). The levels of Strep. mutans and Lactobacillus spp. were low at all test areas, with the maximum mean proportion of Strep. mutans being 1.3 % in Group 4, and the highest mean level of lactobacilli (1.8 %) being found at Group 2 sites. Lower proportions of Veillonella spp. were associated with the two sites which experienced higher amounts of demineralisation.

The mean proportions of Strep. mutans were under 1 % in all Δz groups in Subject D. Strep. sanguis levels were higher in plaques associated with demineralisation of over 500 Δz units, whilst the opposite was observed with Strep. oralis. The mean percentage of Lactobacillus spp. was greater in the highest demineralisation group (15.7 %) than in the other three groups (range 0.8 - 2.9 %).

In Subject E, the mean percentage counts of Strep. mutans were high in all Δz groups, ranging from 19.6 % in Group 2 to 41.4 % in Group 3, and this organism was isolated from each of the 24 plaque samples. Strep. sanguis and Strep. oralis were present in low proportion, or were undetectable, in each group. Lactobacillus spp. increased in proportion from samples associated with minimal demineralisation (4.2 %) to samples from Group 4 lesions (31.8 %), while the percentage of Actinomyces spp. was least in the highest demineralisation category.

The proportion of Strep. mutans in Subject F varied between 0.1 % (Group 3) and 2.4 % in the one sample in Group 4. Lactobacillus spp. decreased with increasing demineralisation, while Actinomyces spp. showed the opposite progression. No obvious trend was seen in relation to the other predominant organisms.

In Subject G, Strep. mutans was isolated from no plaque sample, and, as in Subject F, the mean proportion of Lactobacillus spp. was greatest in Group 1, and decreased thereafter. Veillonella spp. were considerably higher in Δz Groups 1 and 2 (16.0 and 24.9 %, respectively) than in Group 3 (5 %). Similarly, Actinomyces spp. were lowest in the highest demineralisation group.

Results for All Subjects Combined.

The total flora isolated from the plaque associated with each Δz group, for all subjects combined, is shown in Table 4.37.

Strep. mutans increased from a mean percentage count of 5.2 % in Group 1, to a count of 20.0 % in Group 4, with the median values being 0.1 for each of Groups 1 to 3, but 9.5 % for Group 4. The isolation frequency increased throughout the four groups, from 51 % to 100 %, and this was significant at the 1 % level (χ^2 test). The total of the means of the remaining streptococci decreased as the Δz value increased, being approximately 35 % in Group 1 and 17 % in Group 4.

Veillonella spp. accounted for almost the total count of negative cocci isolated, with its highest mean value seen in Group 1 (9.8 %) and

decreasing slightly with increasing demineralisation, the lowest mean count being 5.9 %, in Group 4.

A. odontolyticus and catalase positive A. viscosus / naeslundii showed no pattern with demineralisation group, but both these organisms had their lowest value in the highest Δz group. The catalase negative division of A. viscosus / naeslundii varied little among the demineralisation groups, ranging from 7.0 % (Group 2) to 11.4 % (Group 4).

The mean percentage count of Lactobacillus spp. was similar in Δz Groups 1 -3 (2.4, 3.0 and 2.8 % respectively), but increased to 18.0 % in Group 4. As with Strep. mutans, the median values were 0.1 in Groups 1 to 3, but rose considerably in Group 4, where the median was 12.5 %. The isolation frequencies for this organism were 75.8 %, 65.1 %, 80.8 % and 84.6 % in the four groups. Analysis of variance (shown in Table 3.38.) of the means showed a significantly higher level of lactobacilli in Group 4, compared to each of the other three groups ($p < 0.001$), but χ^2 testing on the isolation frequencies showed no significant differencies.

Bacteroides spp. were present in 42 % of the sites in Groups 1 and 2, 38 % in Group 3, and 8 % in Group 4. The difference in isolation frequency between Group 4 and the other groups was significant at the 5 % level (χ^2 test). The median level was ND in each of the four groups.
The other genera and species, which were only rarely isolated and in low numbers, indicated no trend in relation to demineralisation levels.

The statistical analysis of variance for these results (Table 4.38.) showed little correlation between the percentage counts of the organisms isolated from plaque samples and the amount of demineralisation in associated enamel. Although Strep. mutans increased in mean percentage count throughout the four groups, there was no statistical relationship between the percentage of the organism and the level of demineralisation which occurred. However, while Strep. mutans was present in approximately half the plaque samples associated with minimal mineral loss, in no sample where the mineral loss exceeded 1,000 units was Strep. mutans absent.

Lactobacillus spp. was considerably higher in samples associated with the

greatest amount of mineral loss, compared to the other groups, and this was significant at the 0.1 % level.

4.5.17. Relationship Between Subject Characteristics and *in situ* Demineralisation Levels.

A comparison of the mean levels of demineralisation experienced on the enamel sections of each subject, with the results of the screening tests carried out on the individuals, was undertaken. Only the *in situ* results obtained from NP and SP protocols were used, since Subjects F and G had not participated in experimental runs involving SPM conditions.

Linear regression analysis showed a significant positive correlation ($p < 0.05$) between the DMFS scores of the individuals and the levels of *in situ* mineral loss. Although a positive trend was seen regarding the salivary counts of *Strep. mutans* and *Lactobacillus* spp. and Δz values, this was not significant at the 5 % level. The results regarding salivary flow rate and buffering capacity compared to *in situ* Δz values were equivocal, with one of the two subjects having poor salivary characteristics experiencing the greatest *in situ* demineralisation, while the other experienced a much lower level of mineral loss.

4.5.18. Dietary Assessment of Subjects.

The mean number of exposures per day, of each subject, to fermentable carbohydrate for each experimental run, and an overall mean for all runs combined, is shown in Table 4.39.

The overall means of frequency of intake of the subjects showed a range from 4.1 per day in Subject D to 8.0 per day (Subject F). The average frequency of intake for each subject showed only small differences between experimental runs, with the largest change occurring between Runs 1 and 2 of Subject B, where the values were 7.6 and 5.9 per day, respectively. Analysis of the results, on a day by day basis, showed a slight increase in frequency of intake at week-ends in most subjects, compared to week-days. Some differences in the eating times at week-ends were also observed.

An overall view of the pattern of intake for each subject for a "typical day" is shown in Fig. 4. Differences in the physical form of the potentially cariogenic intake can be seen, with some subjects having a predominantly liquid based challenge whilst in others, foods predominated.

Statistical analysis of the dietary data was performed to determine whether a relationship existed between dietary intake and results of the demineralisation experiments described in this Chapter, for each subject. Linear regression analysis showed no significant correlation between the frequency of fermentable carbohydrate intake and the subjects' *in situ* demineralisation levels.

4.5.19. Microflora of Normal and Abraded Enamel.

The predominant cultivable microflora isolated from plaque overlying normal and abraded enamel sections under NP and SPM conditions are shown in Tables 4.40. and 4.41. Little difference was seen in the proportions or isolation frequencies of the plaque flora from each surface under normal plaque conditions. Both SPM plaques showed a much higher percentage count of *Strep. mutans* than was found in the unstressed plaques (23.3 and 39.4 % compared with 2.3 and 1.0 %), while a decrease was seen in most other genera and species. The proportions of lactobacilli and *Veillonella* spp. were similar in all plaques. A comparison of the SPM plaques obtained from normal and abraded enamel sections showed a higher mean proportion of *Strep. mutans* from the latter, but a lower percentage of positive bacilli.

Tables 4.42. and 4.43. show the mean percentages of the microflora isolated from abraded and non-abraded enamel slabs, under NP and SPM conditions. In NP samples, positive cocci were higher in plaque from non-abraded slabs, (46.4 compared with 24.5 %), while positive bacilli were lower, (36.6 compared with 61.7 %). However, plaques obtained from the two SPM slab surfaces were more similar at the group level although the proportions of streptococcal species varied. Higher levels of *Strep. mutans* were found in both SPM plaques, compared with normal plaques, (11.7 and 7.7 % for SPM; 0.03 % for both NP), whereas the proportion of *Bacteroides* spp. was slightly higher in normal plaques.

Analysis of variance of the results from slabs and sections, found that with both normal plaque and SPM, abrading the enamel surface had no significant effect on the overlying plaque microflora, for any of the groups, genera or species (Table 4.44.). A significant treatment effect was seen, with higher levels of Strep. mutans ($p < 0.001$), and lower levels of Actinomyces spp. ($p < 0.01$), negative bacilli ($p < 0.05$) and Bacteroides spp. ($p < 0.001$) in the SPM plaques compared with the normal plaques. Statistical analysis also showed occasional differences between position and side of enamel on appliance, and experimental runs, but no consistent pattern was observed.

The mean absolute counts of organisms isolated from enamel slabs under NP and SPM conditions are shown in Tables 4.45. and 4.46. As mentioned in Section 4.5.11., a value representing half the minimum detection level was added to each count observed, including those where the organism was not detected, before log transformation. The mean total counts for normal and abraded slabs, under NP conditions, were 7.95 and 7.90 respectively, while the corresponding results from the SPM plaques were 8.17 and 8.00.

Statistical analysis of variance (see Table 4.47.) showed that, for both treatment conditions, abrasion of the enamel surface had no significant effect on the microbial counts, while the inoculation of Strep. mutans and sucrose application, resulted in higher levels of Strep. mutans ($p < 0.001$). To a lesser extent, the counts of Veillonella spp. also increased ($p < 0.05$).

4.5.20. Effect on Abrasion of Enamel Surface on Enamel Demineralisation Parameters.

The demineralisation parameters of normal and abraded enamel, for NP, SPM and both conditions combined, are presented in Tables 4.48. to 4.50. These showed that little demineralisation occurred on any non-abraded surface (mean Δz 99 units (NP), 144 units (SPM)) while the combination of Strep. mutans implantation, with sucrose applications and abrasion of the enamel, produced considerably more mineral loss (mean Δz 1126 units). However, even in this latter category, a wide range of demineralisation was seen, with the Δz ranging from 0 to 4598 units.

The increase in demineralisation occurring in abraded sections, under both NP and SPM conditions combined, is more clearly seen in Table 4.50., and the subsequent Table (4.51.) confirms that abrasion of the enamel surface had a significant effect ($p < 0.05$) on mineral loss. Although a large amount of demineralisation was seen only following the implantation of Strep. mutans, this treatment did not always result in demineralisation (seen in the wide Δz range quoted above), so statistical analysis found that treatment had no significant effect.

4.5.21. Number of Enamel Sites in Each Δz and Lesion Profile Group.

The number of abraded and non-abraded enamel sites under each treatment condition, in each Δz and lesion profile group are given in Tables 4.52. and 4.53. Eight of the nine enamel sites which were non-abraded and received NP conditions were in the lowest group, with respect to both Δz and lesion profile. No non-abraded section experienced demineralisation of more than 500 units. Abrasion of the enamel sections which were to receive normal plaque, resulted in more sites in the higher demineralisation groups, in a pattern similar to non-abraded sections which received SPM conditions. However, sections which were both abraded and inoculated with Strep. mutans showed approximately equal numbers in the lowest and highest demineralisation groups, with relatively few sections having a Δz between 200 and 1,000 units.

4.5.22. Plaque Microflora Associated with Demineralisation Groups.

The predominant cultivable flora isolated from non-abraded enamel surfaces, under both treatment conditions combined, for Δz Groups 1 and 2, is shown in Table 4.54. - as seen from Table 4.52., there were no non-abraded sites in Δz Groups 3 and 4. A lower proportion of Strep. mutans was found in enamel sites in Δz Group 2 (8.3 % compared with 14.1 % in Group 1), but the isolation frequency and median value were greater in the higher demineralisation group, although the number of sites concerned was small.

The percentage distribution of the flora from plaque overlying abraded enamel sites, under both treatment conditions, for all four Δz groups is shown in Table 4.55. The mean percentage of Strep. mutans showed no

association with the amount of demineralisation, the proportions isolated being 20.7, 16.5, 0.2 and 33.0 % in Δz Groups 1 to 4 respectively, but the median was very much higher in Δz Group 4 compared to the other three groups. Only two sites were present in Δz Group 3, and in the other groups large ranges were seen. However, the isolation frequency of this organism increased from 50 % in Group 1 to 100 % in Groups 3 and 4.

Lactobacillus spp. was present in low proportion in all four demineralisation groups, and no pattern was seen with respect to isolation frequencies.

Statistical analysis of variance (see Table 4.56.) of the enamel sites' microflora in each lesion profile and Δz group, showed there was no significant relationship between the proportions of the organisms isolated and the amount of demineralisation on enamel sites.

Table 4.1. Salivary counts of Strep. mutans and Lactobacillus sp. (log_{10} cfu/ml saliva) on three occasions, with mean counts, and DMFS scores of Subjects A - G.

Subject	Strep. mutans	Lact. sp.	DMFS
A	2.62	2.81	22
	3.90	2.75	
	4.23	4.15	
Mean	3.58	3.24	
(SD)[a]	(0.8)	(0.8)	
B	4.57	3.97	19
	4.32	4.34	
	4.68	4.56	
Mean	4.52	4.29	
(SD)	(0.2)	(0.3)	
C	5.92	5.50	24
	5.41	5.23	
	4.97	4.92	
Mean	5.43	5.22	
(SD)	(0.5)	(0.3)	
D	4.89	4.91	28
	4.80	4.84	
	4.68	4.76	
Mean	4.79	4.84	
(SD)	(0.1)	(0.1)	

[a] - SD = Standard Deviation.

Subject	Strep. mutans	Lact. sp.	DMFS
E	6.15	5.95	48
	5.69	5.93	
	5.93	5.48	
Mean	5.92	5.79	
(SD)	(0.2)	(0.3)	
F	5.50	5.45	43
	5.83	4.99	
	4.92	4.94	
Mean	5.42	5.13	
(SD)	(0.5)	(0.3)	
G	5.74	4.89	25
	5.72	5.04	
	5.98	5.62	
Mean	5.81	5.18	
(SD)	(0.1)	(0.4)	

Table 4.1. cont.

Table 4.2. Mixed stimulated salivary flow rate (ml/min), salivary buffering capacity and plaque lactate / acetate ratio before and 6 min following application of 10 % sucrose, on three occasions, for Subjects A - G.

Subject	Flow Rate ml/min	Buff. Cap.	Lact. / Acetate 0	6 min
A	0.7	3.5	0.11	1.14
	0.8	4.2	0.25	0.82
	1.0	5.0	0.12	2.27
Mean	0.8	4.2	0.16	1.41
(SD)	(0.2)	(0.8)	(0.08)	(0.76)
B	1.8	5.5	0.07	1.88
	1.6	5.5	0.27	0.70
	1.9	6.0	0.28	0.97
Mean	1.8	5.7	0.21	1.18
(SD)	(0.2)	(0.3)	(0.12)	(0.62)
C	2.9	5.5	0.39	1.06
	2.1	5.5	0.37	2.56
	2.3	6.0	0.12	0.25
Mean	2.4	5.7	0.29	1.29
(SD)	(0.4)	(0.3)	(0.15)	(1.17)
D	3.5	6.5	0.24	0.98
	3.6	6.5	0.39	1.26
	3.5	6.5	0.17	0.63
Mean	3.5	6.5	0.27	0.96
(SD)	(0.05)	(0)	(0.11)	(0.32)

Subject	Flow Rate ml/min	Buff. Cap.	Lact. / Acetate 0	6 min
E	0.7	6.5	0.44	1.61
	1.0	4.0	0.31	1.46
	0.6	4.0	0.34	2.17
Mean	0.8	4.8	0.36	1.75
(SD)	(0.2)	(1.4)	(0.07)	(0.37)
F	2.3	6.2	0.32	1.22
	2.1	6.0	0.28	1.43
	2.3	6.0	0.25	1.69
Mean	2.2	6.1	0.28	1.45
(SD)	(0.1)	(0.1)	(0.04)	(0.24)
G	3.4	5.5	-	-
	2.3	6.0	-	-
	2.4	6.0	-	-
Mean	2.7	5.8	-	-
(SD)	(0.6)	(0.3)	-	-

Table 4.2. cont.

SECTION - LEFT ALL CONDITIONS

	F[a]	Mean (SD)	Median	Range
+ve cocci	71/72	41.2 (23.5)	42.0	ND[b]- 87.1
S. mutans	45/72	7.6 (15.9)	0.1	ND - 70.9
S. sanguis	50/72	11.0 (13.6)	6.0	ND - 61.3
S. oralis	48/72	16.1 (19.7)	8.6	ND - 76.9
-ve cocci	51/72	8.3 (9.9)	4.0	ND - 37.1
Veillonella	50/72	7.7 (9.4)	3.6	ND - 94.7
+ve bacilli	72/72	45.0 (24.4)	41.4	0.1 - 96.6
Actinomyces	69/72	36.2 (24.8)	32.5	ND - 94.7
Lactobacillus	57/72	5.1 (11.6)	0.1	ND - 49.0
-ve bacilli	41/72	5.5 (7.7)	3.2	ND - 31.2
Bacteroides	22/72	2.3 (5.1)	ND	ND - 28.6

SECTION - RIGHT ALL CONDITIONS

	F	Mean (SD)	Median	Range
+ve cocci	72/72	40.0 (22.9)	37.2	0.1 - 91.3
S. mutans	46/72	8.3 (21.2)	0.1	ND - 88.3
S. sanguis	42/72	9.9 (12.8)	4.0	ND - 29.0
S. oralis	52/72	16.3 (17.0)	11.9	ND - 53.8
-ve cocci	55/72	9.6 (10.5)	6.6	ND - 41.7
Veillonella	51/72	8.8 (10.5)	5.6	ND - 39.3
+ve bacilli	72/72	43.0 (25.6)	41.2	0.5 -100.0
Actinomyces	72/72	36.2 (23.2)	34.0	0.5 - 82.8
Lactobacillus	50/72	2.9 (8.0)	0.1	ND - 12.5
-ve bacilli	47/72	7.2 (8.2)	4.0	ND - 40.0
Bacteroides	33/72	3.9 (5.7)	ND	ND - 11.1

[a] - F = Isolation Frequency ; [b] - ND = Not Detectable.

Table 4.3. % predominant cultivable plaque microflora isolated from enamel sections on left and right sides of appliance, under all treatment conditions, for all Subjects combined.

SLAB - LEFT ALL CONDITIONS

	F	Mean (SD)	Median	Range
+ve cocci	68/69	38.5 (22.3)	36.0	ND - 86.7
S. mutans	50/69	9.8 (19.2)	0.1	ND - 66.7
S. sanguis	38/69	7.6 (11.5)	3.0	ND - 68.0
S. oralis	56/69	16.1 (13.9)	13.3	ND - 52.0
-ve cocci	56/69	8.9 (8.7)	7.4	ND - 41.4
Veillonella	54/69	8.3 (8.7)	6.2	ND - 41.4
+ve bacilli	69/69	45.4 (24.2)	44.8	0.1 - 95.6
Actinomyces	69/69	41.8 (23.0)	40.6	0.1 - 95.6
Lactobacillus	57/69	1.5 (3.5)	0.1	ND - 21.6
-ve bacilli	46/69	7.1 (8.2)	6.2	ND - 52.0
Bacteroides	34/69	3.8 (6.0)	ND	ND - 36.0

SLAB - RIGHT ALL CONDITIONS

	F	Mean (SD)	Median	Range
+ve cocci	69/69	34.6 (21.6)	32.0	0.1 - 83.9
S. mutans	41/69	6.4 (17.2)	0.1	ND - 77.4
S. sanguis	54/69	12.0 (10.9)	10.0	ND - 50.0
S. oralis	56/69	12.5 (12.0)	10.0	ND - 48.1
-ve cocci	52/69	6.6 (6.8)	5.6	ND - 36.4
Veillonella	51/69	6.0 (6.8)	4.4	ND - 36.4
+ve bacilli	69/69	49.7 (21.4)	50.0	3.2 - 94.7
Actinomyces	69/69	46.3 (20.5)	45.8	3.2 - 89.4
Lactobacillus	37/69	2.0 (5.9)	0.1	ND - 44.4
-ve bacilli	49/69	9.1 (10.9)	7.1	ND - 60.0
Bacteroides	40/69	5.1 (8.2)	3.3	ND - 56.0

Table 4.4. % predominant cultivable plaque microflora isolated from enamel slabs on left and right sides of appliance, under all treatment conditions, for all Subjects combined.

Table 4.5. % predominant cultivable plaque microflora isolated from each enamel section position, under all treatment conditions, for all Subjects combined.

SECTION - MESIAL SUPERIOR
ALL CONDITIONS

	F	Mean (SD)	Median	Range
+ve cocci	36/36	43.6 (20.8)	42.8	3.4 - 85.7
S. mutans	23/36	8.4 (19.2)	0.1	ND - 73.0
S. sanguis	28/36	13.4 (16.5)	7.2	ND - 61.3
S. oralis	27/36	14.5 (15.8)	9.7	ND - 53.8
-ve cocci	29/36	7.8 (8.3)	5.7	ND - 32.0
Veillonella	28/36	7.0 (7.4)	5.0	ND - 31.0
+ve bacilli	36/36	41.5 (21.8)	39.8	6.1 - 96.6
Actinomyces	35/36	33.5 (19.9)	31.6	ND - 79.3
Lactobacillus	29/36	2.9 (7.4)	0.1	ND - 40.0
-ve bacilli	20/36	6.8 (10.4)	1.6	ND - 40.0
Bacteroides	12/36	2.7 (5.0)	ND	ND - 20.0

SECTION - MESIAL INFERIOR
ALL CONDITIONS

	F	Mean (SD)	Median	Range
+ve cocci	35/36	43.6 (24.6)	38.0	ND - 87.1
S. mutans	22/36	8.3 (21.3)	0.1	ND - 75.3
S. sanguis	23/36	9.1 (9.9)	5.2	ND - 29.6
S. oralis	26/36	18.9 (19.6)	14.3	ND - 63.6
-ve cocci	26/36	10.2 (12.0)	4.8	ND - 40.4
Veillonella	24/36	9.3 (12.2)	3.9	ND - 40.4
+ve bacilli	36/36	40.1 (26.0)	40.8	3.0 - 87.5
Actinomyces	36/36	35.3 (24.9)	33.4	2.0 - 87.5
Lactobacillus	21/36	2.3 (8.3)	0.1	ND - 49.0
-ve bacilli	22/36	6.1 (7.0)	4.2	ND - 31.2
Bacteroides	14/36	2.6 (4.1)	ND	ND - 14.8

SECTION - DISTAL SUPERIOR
ALL CONDITIONS

	F	Mean (SD)	Median	Range
+ve cocci	36/36	36.2 (23.3)	39.1	0.1 - 78.3
S. mutans	25/36	6.6 (14.2)	0.1	ND - 48.0
S. sanguis	19/36	8.2 (11.6)	4.0	ND - 45.7
S. oralis	25/36	16.8 (19.6)	10.6	ND - 69.6
-ve cocci	24/36	7.8 (9.3)	4.6	ND - 37.1
Veillonella	24/36	7.4 (8.7)	4.6	ND - 34.3
+ve bacilli	36/36	49.2 (24.8)	49.7	12.5 - 93.1
Actinomyces	35/36	37.4 (24.5)	31.5	ND - 93.0
Lactobacillus	31/36	6.8 (13.3)	0.1	ND - 48.9
-ve bacilli	23/36	6.6 (7.7)	4.2	ND - 28.6
Bacteroides	14/36	4.0 (7.2)	ND	ND - 28.6

SECTION - DISTAL INFERIOR
ALL CONDITIONS

	F	Mean (SD)	Median	Range
+ve cocci	34/36	38.8 (23.8)	35.2	ND - 91.3
S. mutans	21/36	8.4 (19.4)	0.1	ND - 88.3
S. sanguis	22/36	11.2 (13.8)	7.4	ND - 60.7
S. oralis	22/36	14.8 (18.4)	7.7	ND - 76.9
-ve cocci	27/36	10.0 (11.0)	5.7	ND - 41.7
Veillonella	25/36	9.4 (11.0)	4.6	ND - 41.7
+ve bacilli	36/36	45.2 (27.0)	41.9	0.1 -100.0
Actinomyces	34/36	38.6 (26.4)	36.4	ND -100.0
Lactobacillus	26/36	4.2 (9.9)	0.1	ND - 46.4
-ve bacilli	23/36	6.0 (6.6)	3.8	ND - 24.3
Bacteroides	15/36	3.0 (5.3)	ND	ND - 24.3

Table 4.5. cont.

Table 4.6. % predominant cultivable plaque microflora isolated from each enamel slab position, under all treatment conditions, for all Subjects combined.

SLAB - MESIAL SUPERIOR
ALL CONDITIONS

	F	Mean (SD)	Median	Range
+ve cocci	36/36	34.7 (23.1)	30.0	0.1 - 77.1
S. mutans	20/36	7.1 (17.4)	0.1	ND - 62.3
S. sanguis	24/36	11.6 (12.2)	10.2	ND - 42.0
S. oralis	27/36	10.4 (10.0)	8.2	ND - 35.7
-ve cocci	25/36	7.7 (9.5)	4.4	ND - 36.4
Veillonella	23/36	7.2 (9.6)	4.1	ND - 36.4
+ve bacilli	36/36	47.0 (26.3)	44.9	0.1 - 95.6
Actinomyces	36/36	43.3 (25.4)	37.0	0.1 - 95.6
Lactobacillus	27/36	2.1 (7.6)	0.1	ND - 44.4
-ve bacilli	25/36	10.7 (13.9)	6.4	ND - 60.0
Bacteroides	17/36	6.0 (11.5)	ND	ND - 56.0

SLAB - MESIAL INFERIOR
ALL CONDITIONS

	F	Mean (SD)	Median	Range
+ve cocci	36/36	40.4 (23.4)	36.6	4.3 - 78.8
S. mutans	26/36	7.9 (18.5)	0.1	ND - 66.7
S. sanguis	19/36	9.5 (15.2)	3.2	ND - 68.0
S. oralis	29/36	17.9 (15.7)	13.5	ND - 48.1
-ve cocci	25/36	6.9 (6.1)	7.0	ND - 24.3
Veillonella	24/36	6.5 (5.9)	7.0	ND - 24.3
+ve bacilli	36/36	44.9 (23.6)	41.7	0.1 - 87.0
Actinomyces	36/36	42.0 (22.7)	40.0	0.1 - 81.2
Lactobacillus	22/36	1.7 (4.3)	0.1	ND - 21.6
-ve bacilli	26/36	7.8 (9.0)	7.1	ND - 46.4
Bacteroides	18/36	3.3 (4.0)	1.2	ND - 13.5

SLAB - DISTAL SUPERIOR
ALL CONDITIONS

	F	Mean (SD)	Median	Range
+ve cocci	35/35	34.2 (19.5)	30.0	3.8 - 76.0
S. mutans	26/35	7.7 (16.5)	0.1	ND - 66.0
S. sanguis	25/35	8.0 (7.2)	8.3	ND - 26.3
S. oralis	29/35	15.0 (12.3)	12.5	ND - 52.0
-ve cocci	30/35	6.9 (5.3)	5.6	ND - 25.0
Veillonella	30/35	6.3 (5.1)	5.0	ND - 25.0
+ve bacilli	35/35	51.2 (20.2)	55.6	7.7 - 92.3
Actinomyces	35/35	47.9 (19.6)	52.2	7.7 - 88.5
Lactobacillus	27/35	1.4 (2.6)	0.1	ND - 23.5
-ve bacilli	24/35	7.6 (7.1)	6.2	ND - 23.5
Bacteroides	21/35	4.6 (5.9)	4.0	ND - 23.5

SLAB - DISTAL INFERIOR
ALL CONDITIONS

	F	Mean (SD)	Median	Range
+ve cocci	31/31	36.8 (21.8)	34.8	6.9 - 86.7
S. mutans	18/31	10.0 (21.3)	0.1	ND - 77.4
S. sanguis	24/31	10.0 (9.0)	8.0	ND - 28.6
S. oralis	27/31	14.0 (13.1)	8.7	ND - 46.6
-ve cocci	28/31	9.8 (9.8)	8.0	ND - 41.4
Veillonella	28/31	8.9 (9.9)	5.0	ND - 41.4
+ve bacilli	31/31	47.2 (21.1)	50.0	0.1 - 87.5
Actinomyces	30/31	43.0 (18.9)	44.0	ND - 79.2
Lactobacillus	25/31	1.7 (2.8)	0.1	ND - 9.6
-ve bacilli	20/31	6.0 (6.2)	5.7	ND - 27.3
Bacteroides	18/31	3.9 (4.3)	3.3	ND - 15.0

Table 4.6. cont.

Table 4.7. % predominant cultivable plaque microflora isolated from enamel sections, under Protocol I (NP), for Subjects A - G.

SECTION - NORMAL PLAQUE
SUBJECT A

	F	Mean (SD)	Median	Range
+ve cocci	8/8	41.7 (21.7)	42.4	14.3 - 71.4
S. mutans	2/8	0.02 (0.05)	ND	ND - 0.1
S. sanguis	7/8	20.4 (14.4)	17.2	ND - 45.7
S. oralis	7/8	16.6 (13.9)	15.8	ND - 38.9
-ve cocci	5/8	7.1 (10.8)	2.4	ND - 26.7
Veillonella	5/8	7.1 (10.8)	2.4	ND - 26.7
+ve bacilli	8/8	47.4 (21.3)	42.1	25.7 - 80.0
Actinomyces	8/8	43.5 (24.2)	42.1	6.1 - 77.1
Lactobacillus	3/8	0.8 (2.2)	ND	ND - 6.1
-ve bacilli	6/8	3.7 (3.9)	2.4	ND - 11.1
Bacteroides	3/8	2.3 (4.1)	ND	ND - 11.1

SECTION - NORMAL PLAQUE
SUBJECT B

	F	Mean (SD)	Median	Range
+ve cocci	8/8	36.3 (9.9)	39.2	16.1 - 45.6
S. mutans	1/8	0.01 (0.04)	ND	ND - 0.1
S. sanguis	7/8	9.5 (8.0)	7.0	ND - 24.1
S. oralis	7/8	23.4 (13.6)	28.8	ND - 37.1
-ve cocci	8/8	17.6 (10.8)	12.5	6.4 - 37.1
Veillonella	8/8	15.9 (11.0)	10.1	6.4 - 34.3
+ve bacilli	8/8	37.6 (15.7)	33.7	21.7 - 74.2
Actinomyces	8/8	36.0 (15.0)	33.0	21.7 - 70.9
Lactobacillus	3/8	0.04 (0.05)	ND	ND - 0.1
-ve bacilli	7/8	8.4 (6.9)	7.4	ND - 19.6
Bacteroides	5/8	5.1 (5.8)	3.1	ND - 15.2

SECTION - NORMAL PLAQUE
SUBJECT C

	F	Mean (SD)	Median	Range
+ve cocci	8/8	54.7 (15.2)	50.9	32.2 - 82.4
S. mutans	5/8	0.2 (0.5)	0.1	ND - 1.5
S. sanguis	8/8	27.5 (17.0)	22.7	12.9 - 60.6
S. oralis	8/8	20.2 (11.7)	21.4	3.8 - 33.3
-ve cocci	7/8	10.9 (10.9)	7.0	ND - 29.6
Veillonella	7/8	10.9 (10.9)	7.0	ND - 29.6
+ve bacilli	8/8	27.8 (17.9)	25.3	5.9 - 53.1
Actinomyces	8/8	25.0 (15.4)	25.3	5.9 - 46.2
Lactobacillus	8/8	0.1 (0.05)	0.1	0.1 - 0.2
-ve bacilli	4/8	7.2 (8.4)	4.0	ND - 19.4
Bacteroides	1/8	0.7 (2.1)	ND	ND - 5.9

SECTION - NORMAL PLAQUE
SUBJECT D

	F	Mean (SD)	Median	Range
+ve cocci	8/8	28.6 (20.7)	24.6	1.2 - 60.5
S. mutans	5/8	0.5 (1.0)	0.1	ND - 2.7
S. sanguis	5/8	5.5 (4.7)	7.3	ND - 10.7
S. oralis	6/8	18.7 (10.9)	11.0	ND - 50.0
-ve cocci	4/8	2.6 (3.2)	1.3	ND - 7.7
Veillonella	4/8	2.2 (2.8)	1.3	ND - 7.7
+ve bacilli	8/8	66.5 (22.2)	70.2	34.6 -100.0
Actinomyces	8/8	65.3 (23.0)	70.2	30.7 -100.0
Lactobacillus	5/8	1.3 (1.7)	0.1	ND - 3.8
-ve bacilli	2/8	2.2 (5.0)	ND	ND - 14.3
Bacteroides	2/8	1.3 (2.6)	ND	ND - 7.1

Table 4.7. cont.

SECTION - NORMAL PLAQUE
SUBJECT E

	F	Mean (SD)	Median	Range
+ve cocci	8/8	35.3 (19.2)	41.6	1.4 - 54.2
S. mutans	8/8	21.2 (18.4)	24.6	0.1 - 41.7
S. sanguis	4/8	5.6 (7.8)	1.8	ND - 20.0
S. oralis	3/8	5.2 (9.9)	ND	ND - 28.0
-ve cocci	4/8	6.7 (11.1)	1.8	ND - 32.0
Veillonella	1/8	2.0 (5.7)	ND	ND - 16.0
+ve bacilli	8/8	51.5 (23.4)	47.2	20.0 - 87.5
Actinomyces	7/8	43.4 (29.0)	39.9	ND - 87.5
Lactobacillus	8/8	5.4 (11.2)	0.1	0.1 - 32.1
-ve bacilli	4/8	6.5 (9.9)	2.1	ND - 28.6
Bacteroides	4/8	6.5 (9.9)	2.1	ND - 28.6

SECTION - NORMAL PLAQUE
SUBJECT F

	F	Mean (SD)	Median	Range
+ve cocci	8/8	19.4 (24.4)	11.9	3.4 - 76.9
S. mutans	1/8	0.01 (0.04)	ND	ND - 0.1
S. sanguis	5/8	4.7 (5.0)	3.4	ND - 12.5
S. oralis	3/8	11.6 (26.7)	ND	ND - 76.9
-ve cocci	5/8	6.8 (6.8)	6.0	ND - 17.4
Veillonella	4/8	5.1 (6.5)	2.1	ND - 17.4
+ve bacilli	8/8	58.0 (27.6)	64.6	0.1 - 89.3
Actinomyces	7/8	36.3 (22.4)	35.4	ND - 65.2
Lactobacillus	6/8	8.9 (16.1)	0.1	ND - 46.4
-ve bacilli	8/8	15.8 (10.4)	14.2	3.6 - 31.2
Bacteroides	4/8	3.2 (4.8)	1.4	ND - 13.8

Table 4.7. cont.

SECTION - NORMAL PLAQUE
SUBJECT G

	F	Mean (SD)	Median	Range
+ve cocci	4/4	49.2 (6.0)	48.4	42.8 - 57.1
S. mutans	0/4	ND	ND	ND
S. sanguis	4/4	28.2 (5.0)	29.5	21.4 - 32.5
S. oralis	3/4	9.6 (9.1)	10.4	ND - 17.5
-ve cocci	4/4	25.6 (14.8)	28.6	5.0 - 40.4
Veillonella	4/4	25.6 (14.8)	28.6	5.0 - 40.4
+ve bacilli	4/4	21.3 (12.8)	19.6	8.5 - 37.5
Actinomyces	4/4	11.1 (10.8)	8.4	2.5 - 25.0
Lactobacillus	4/4	0.6 (1.0)	0.1	0.1 - 2.1
-ve bacilli	3/4	3.8 (3.1)	3.9	ND - 7.5
Bacteroides	3/4	2.0 (1.5)	2.3	ND - 3.6

Table 4.7. cont.

Table 4.8. % predominant cultivable plaque microflora isolated from enamel sections, under Protocol II (SP), for Subjects A - G.

SECTION - SUCROSE PLAQUE
SUBJECT A

	F	Mean (SD)	Median	Range
+ve cocci	8/8	54.0 (21.1)	54.6	31.6 - 86.9
S. mutans	5/8	0.1 (0.05)	0.1	ND - 0.1
S. sanguis	4/8	10.6 (14.1)	1.8	ND - 33.3
S. oralis	6/8	30.4 (24.9)	38.2	ND - 59.6
-ve cocci	8/8	10.0 (13.3)	4.5	2.7 - 41.7
Veillonella	8/8	10.0 (13.3)	4.5	2.7 - 41.7
+ve bacilli	8/8	22.7 (18.9)	17.8	3.0 - 63.2
Actinomyces	8/8	16.8 (9.1)	17.8	3.0 - 31.6
Lactobacillus	6/8	0.6 (1.4)	0.1	ND - 4.0
-ve bacilli	7/8	12.6 (13.5)	7.7	ND - 40.0
Bacteroides	4/8	8.4 (10.0)	4.2	ND - 24.3

SECTION - SUCROSE PLAQUE
SUBJECT B

	F	Mean (SD)	Median	Range
+ve cocci	8/8	24.6 (11.2)	25.8	7.3 - 40.0
S. mutans	3/8	1.9 (5.3)	ND	ND - 15.0
S. sanguis	5/8	4.7 (4.9)	3.7	ND - 12.0
S. oralis	8/8	18.0 (9.1)	18.6	2.4 - 33.3
-ve cocci	6/8	7.5 (5.9)	9.7	ND - 13.8
Veillonella	6/8	6.1 (5.2)	5.8	ND - 12.5
+ve bacilli	8/8	55.0 (15.5)	49.2	41.7 - 87.8
Actinomyces	8/8	50.6 (15.6)	46.4	39.1 - 87.8
Lactobacillus	2/8	0.02(0.05)	ND	ND - 0.1
-ve bacilli	8/8	11.1 (4.4)	11.2	4.9 - 18.5
Bacteroides	8/8	9.0 (5.6)	7.6	2.5 - 18.5

SECTION - SUCROSE PLAQUE
SUBJECT C

	F	Mean (SD)	Median	Range
+ve cocci	8/8	56.2 (17.6)	59.4	7.8 - 78.3
S. mutans	7/8	0.1 (0.04)	0.1	ND - 0.1
S. sanguis	4/8	8.7 (12.1)	2.2	ND - 29.4
S. oralis	8/8	46.4 (16.3)	42.2	27.8 - 69.6
-ve cocci	4/8	4.1 (5.8)	1.4	ND - 14.3
Veillonella	4/8	4.1 (5.8)	1.4	ND - 14.3
+ve bacilli	8/8	27.2 (20.2)	14.8	10.8 - 55.6
Actinomyces	8/8	27.2 (20.2)	14.8	10.8 - 55.6
Lactobacillus	5/8	0.1 (0.05)	0.1	ND - 0.1
-ve bacilli	7/8	12.5 (9.8)	12.6	ND - 29.2
Bacteroides	1/8	2.6 (7.4)	ND	ND - 20.8

SECTION - SUCROSE PLAQUE
SUBJECT D

	F	Mean (SD)	Median	Range
+ve cocci	8/8	43.6 (22.9)	41.4	6.9 - 82.1
S. mutans	5/8	0.2 (0.4)	0.1	ND - 1.1
S. sanguis	8/8	27.5 (21.6)	17.3	6.9 - 61.3
S. oralis	6/8	9.7 (9.4)	8.8	ND - 27.6
-ve cocci	4/8	8.1 (9.7)	3.8	ND - 23.1
Veillonella	4/8	8.1 (9.7)	3.8	ND - 23.1
+ve bacilli	8/8	46.5 (24.0)	42.3	10.7 - 93.1
Actinomyces	8/8	40.1 (24.2)	31.6	10.7 - 93.0
Lactobacillus	6/8	0.9 (1.6)	0.1	ND - 3.7
-ve bacilli	2/8	1.8 (3.4)	ND	ND - 7.7
Bacteroides	1/8	1.0 (2.7)	ND	ND - 7.7

Table 4.8. cont.

SECTION - SUCROSE PLAQUE
SUBJECT E

	F	Mean (SD)	Median	Range
+ve cocci	8/8	22.6 (12.5)	22.6	3.6 - 37.0
S. mutans	8/8	11.9 (12.5)	6.8	0.1 - 32.3
S. sanguis	2/8	1.0 (1.8)	ND	ND - 4.0
S. oralis	2/8	3.1 (7.0)	ND	ND - 20.0
-ve cocci	5/8	12.6 (12.4)	11.9	ND - 29.0
Veillonella	5/8	12.0 (11.6)	11.9	ND - 29.0
+ve bacilli	8/8	61.9 (17.7)	59.9	35.5 - 92.8
Actinomyces	8/8	54.4 (11.8)	56.2	35.4 - 71.4
Lactobacillus	7/8	5.7 (7.5)	3.1	ND - 20.0
-ve bacilli	4/8	2.8 (4.1)	1.6	ND - 12.0
Bacteroides	2/8	1.4 (2.9)	ND	ND - 8.0

SECTION - SUCROSE PLAQUE
SUBJECT F

	F	Mean (SD)	Median	Range
+ve cocci	8/8	36.6 (26.2)	29.9	6.9 - 77.3
S. mutans	6/8	1.6 (2.4)	0.1	ND - 5.6
S. sanguis	4/8	6.8 (8.9)	3.1	ND - 24.4
S. oralis	5/8	20.5 (25.7)	10.8	ND - 63.6
-ve cocci	7/8	4.8 (4.0)	3.8	ND - 13.8
Veillonella	7/8	4.4 (3.0)	3.8	ND - 10.3
+ve bacilli	8/8	47.3 (31.2)	62.8	5.0 - 80.6
Actinomyces	8/8	35.4 (26.0)	31.1	5.0 - 75.0
Lactobacillus	5/8	6.1 (14.5)	0.1	ND - 41.4
-ve bacilli	7/8	11.3 (10.0)	7.8	ND - 29.4
Bacteroides	3/8	2.8 (5.2)	ND	ND - 15.0

Table 4.8. cont.

SECTION - SUCROSE PLAQUE
SUBJECT G

	F	Mean (SD)	Median	Range
+ve cocci	4/4	49.2 (6.0)	77.8	54.8 - 87.1
S. mutans	0/4	ND	ND	ND
S. sanguis	4/4	14.6 (12.2)	10.8	4.6 - 32.2
S. oralis	0/4	ND	ND	ND
-ve cocci	3/4	10.3 (11.1)	7.7	ND - 25.8
Veillonella	3/4	10.3 (11.1)	7.7	ND - 25.8
+ve bacilli	4/4	15.4 (12.0)	14.0	3.2 - 30.2
Actinomyces	4/4	6.8 (5.0)	5.9	2.3 - 12.9
Lactobacillus	4/4	7.8 (13.5)	1.6	0.1 - 27.9
-ve bacilli	0/4	ND	ND	ND
Bacteroides	0/4	ND	ND	ND

Table 4.8. cont.

Table 4.9. % predominant cultivable plaque microflora isolated from enamel sections, under Protocol III (SPM), for Subjects A - E.

SECTION - SPM
SUBJECT A

	F	Mean (SD)	Median	Range
+ve cocci	8/8	65.8 (17.4)	64.8	35.7 - 91.3
S. mutans	8/8	55.3 (22.4)	52.4	19.6 - 88.3
S. sanguis	6/8	3.3 (2.6)	3.8	ND - 7.7
S. oralis	6/8	5.2 (4.7)	4.4	ND - 12.3
-ve cocci	7/8	5.3 (5.7)	3.2	ND - 17.8
Veillonella	7/8	5.3 (5.7)	3.2	ND - 17.8
+ve bacilli	8/8	25.3 (17.8)	27.0	0.5 - 55.4
Actinomyces	8/8	18.7 (12.8)	22.0	0.5 - 33.9
Lactobacillus	7/8	3.2 (5.8)	0.1	ND - 12.5
-ve bacilli	5/8	2.9 (3.8)	1.7	ND - 10.8
Bacteroides	3/8	0.5 (0.8)	ND	ND - 1.8

SECTION - SPM
SUBJECT B

	F	Mean (SD)	Median	Range
+ve cocci	7/8	24.3 (17.3)	29.0	ND - 91.3
S. mutans	4/8	0.05 (0.05)	0.05	ND - 0.1
S. sanguis	6/8	11.8 (13.0)	5.8	ND - 35.3
S. oralis	6/8	11.8 (8.4)	12.2	ND - 23.5
-ve cocci	5/8	10.2 (12.9)	9.4	ND - 39.3
Veillonella	5/8	10.2 (12.9)	9.4	ND - 39.3
+ve bacilli	8/8	59.6 (24.6)	60.8	21.4 - 94.7
Actinomyces	8/8	57.0 (23.6)	56.5	21.4 - 94.7
Lactobacillus	6/8	1.5 (3.0)	0.1	ND - 8.3
-ve bacilli	6/8	5.9 (5.0)	4.4	ND - 11.8
Bacteroides	5/8	5.4 (5.4)	4.4	ND - 11.8

SECTION - SPM
SUBJECT C

	F	Mean (SD)	Median	Range
+ve cocci	8/8	58.1 (10.7)	61.0	37.5 - 71.8
S. mutans	7/8	0.2 (0.4)	0.1	ND - 1.3
S. sanguis	7/8	17.9 (12.2)	17.0	ND - 35.0
S. oralis	8/8	34.5 (16.0)	30.2	16.1 - 59.1
-ve cocci	7/8	14.2 (11.6)	13.2	ND - 38.7
Veillonella	7/8	14.2 (11.6)	13.2	ND - 38.7
+ve bacilli	8/8	25.2 (10.2)	23.8	12.9 - 45.8
Actinomyces	8/8	23.6 (9.9)	23.8	12.9 - 41.7
Lactobacillus	6/8	1.7 (3.2)	0.1	ND - 8.6
-ve bacilli	2/8	2.5 (4.6)	ND	ND - 11.1
Bacteroides	0/8	ND	ND	ND

SECTION - SPM
SUBJECT D

	F	Mean (SD)	Median	Range
+ve cocci	8/8	15.2 (13.0)	11.8	2.9 - 34.4
S.mutans	8/8	0.1 (0.0)	0.1	0.1
S. sanguis	2/8	1.2 (2.6)	ND	ND - 7.4
S. oralis	7/8	11.8 (11.1)	10.0	ND - 34.3
-ve cocci	7/8	11.2 (11.8)	6.2	ND - 29.4
Veillonella	6/8	10.1 (11.1)	6.2	ND - 29.4
+ve bacilli	8/8	68.5 (18.6)	67.2	48.1 - 96.6
Actinomyces	8/8	54.7 (17.5)	56.6	33.3 - 79.3
Lactobacillus	8/8	10.8 (16.0)	5.4	0.1 - 48.9
-ve bacilli	5/8	5.0 (4.9)	5.2	ND - 12.5
Bacteroides	5/8	3.6 (3.9)	3.4	ND - 11.1

Table 4.9. cont.

SECTION - SPM
SUBJECT E

	F	Mean (SD)	Median	Range
+ve cocci	8/8	50.8 (20.9)	53.2	12.2 - 73.9
S. mutans	8/8	49.4 (20.7)	51.2	12.2 - 73.9
S. sanguis	0/8	ND	ND	ND
S. oralis	1/8	0.4 (1.0)	ND	ND - 2.9
-ve cocci	6/8	3.5 (2.9)	3.6	ND - 8.8
Veillonella	6/8	3.5 (2.9)	3.6	ND - 8.8
+ve bacilli	8/8	45.1 (20.7)	40.0	23.3 - 83.7
Actinomyces	7/8	14.5 (14.8)	11.4	ND - 40.0
Lactobacillus	8/8	21.5 (20.5)	16.5	0.1 - 49.0
-ve bacilli	1/8	0.5 (1.4)	ND	ND - 4.0
Bacteroides	1/8	0.5 (1.4)	ND	ND - 4.0

Table 4.9. cont.

Table 4.10. % predominant cultivable plaque microflora isolated from enamel sections, under each treatment condition, for five Subjects, A - E, combined.

SECTION - NORMAL PLAQUE
SUBJECTS A - E

	F	Mean (SD)	Median	Range
+ve cocci	40/40	39.3 (19.1)	41.4	1.2 - 82.4
S. mutans	21/40	4.4 (11.6)	0.1	ND - 41.7
S. sanguis	31/40	13.7 (13.9)	11.8	ND - 60.6
S. oralis	31/40	16.8 (15.1)	12.1	ND - 50.0
-ve cocci	28/40	9.0 (10.6)	4.7	ND - 37.1
Veillonella	25/40	7.6 (10.0)	3.4	ND - 34.3
+ve bacilli	40/40	46.2 (23.3)	40.0	5.9 -100.0
Actinomyces	39/40	42.6 (24.7)	36.5	ND -100.0
Lactobacillus	27/40	1.5 (5.3)	0.1	ND - 32.1
-ve bacilli	23/40	5.6 (7.2)	2.8	ND - 28.6
Bacteroides	15/40	3.2 (5.8)	ND	ND - 28.6

SECTION - SUCROSE PLAQUE
SUBJECTS A - E

	F	Mean (SD)	Median	Range
+ve cocci	40/40	40.2 (22.0)	34.0	3.6 - 86.9
S. mutans	28/40	2.8 (7.4)	0.1	ND - 32.3
S. sanguis	23/40	10.5 (15.4)	3.8	ND - 61.3
S. oralis	30/40	21.5 (21.0)	17.5	ND - 69.6
-ve cocci	27/40	8.4 (9.8)	4.8	ND - 41.7
Veillonella	27/40	8.0 (9.6)	4.0	ND - 41.7
+ve bacilli	40/40	42.7 (24.1)	44.8	3.0 - 93.1
Actinomyces	40/40	37.8 (21.6)	41.3	3.0 - 93.0
Lactobacillus	26/40	1.4 (3.9)	0.1	ND - 20.0
-ve bacilli	28/40	8.2 (9.0)	6.7	ND - 40.0
Bacteroides	16/40	4.5 (7.0)	ND	ND - 24.3

SECTION - SPM
SUBJECTS A - E

	F	Mean (SD)	Median	Range
+ve cocci	39/40	42.9 (25.2)	45.6	ND - 91.3
S. mutans	35/40	21.0 (29.0)	0.1	ND - 88.3
S. sanguis	21/40	6.9 (10.4)	2.2	ND - 35.3
S. oralis	28/40	12.7 (15.0)	9.2	ND - 59.1
-ve cocci	32/40	8.9 (10.1)	5.0	ND - 39.3
Veillonella	31/40	8.7 (9.9)	5.0	ND - 39.3
+ve bacilli	40/40	44.7 (25.3)	40.0	0.5 - 96.6
Actinomyces	39/40	33.7 (24.2)	29.1	ND - 94.7
Lactobacillus	35/40	7.7 (13.8)	0.2	ND - 49.0
-ve bacilli	19/40	3.4 (4.4)	ND	ND - 12.5
Bacteroides	14/40	2.0 (3.6)	ND	ND - 11.8

Table 4.10. cont.

Table 4.11. % total cultivable plaque microflora isolated from enamel sections, under each treatment condition, for all Subjects combined.

SECTION - NORMAL PLAQUE

	F	Mean (SD)	Median	Range
+ve cocci	52/52	37.0 (20.7)	39.6	1.2 - 82.4
S. mutans	22/52	3.4 (10.3)	ND	ND - 41.7
S. sanguis	40/52	13.4 (13.5)	10.9	ND - 60.6
S. oralis	37/52	15.5 (16.9)	9.8	ND - 76.9
S. salivarius	8/52	1.9 (6.0)	ND	ND - 35.3
S. milleri	2/52	0.2 (1.1)	ND	ND - 7.4
Other Strep.	5/52	0.9 (3.0)	ND	ND - 12.5
ANO_2 Strep.	13/52	1.8 (4.6)	ND	ND - 27.7
Micrococcus/Staph.	1/52	0.04 (0.3)	ND	ND - 2.1
-ve cocci	37/52	9.9 (11.3)	5.8	ND - 40.4
Veillonella	33/52	8.6 (11.0)	3.7	ND - 40.4
Neisseria	11/52	1.3 (3.2)	ND	ND - 16.0
+ve bacilli	52/52	46.1 (24.5)	40.8	0.1 -100.0
A. odontolyticus	40/52	18.2 (15.8)	18.2	ND - 68.8
A. v/n - cat. +ve	30/52	10.4 (16.0)	2.7	ND - 66.7
A. v/n - cat. -ve	29/52	8.7 (12.4)	3.6	ND - 41.0
Other Actinomyces	9/52	1.9 (5.2)	ND	ND - 21.7
Lactobacillus	37/52	2.6 (8.0)	0.1	ND - 46.4
Bifidobacterium	10/52	3.1 (8.2)	ND	ND - 37.8
Propionibacterium	1/52	0.1 (0.6)	ND	ND - 4.2
Arachnia	1/52	0.1 (0.9)	ND	ND - 6.2
Eubacterium	5/52	0.4 (1.4)	ND	ND - 6.2
Unidentified	4/52	0.5 (2.0)	ND	ND - 12.5
-ve bacilli	34/52	7.0 (8.3)	3.6	ND - 31.2
Bacteroides	22/52	3.1 (5.4)	ND	ND - 28.6
Fusobacterium	7/52	0.4 (1.2)	ND	ND - 4.3
Capnocytophaga	17/52	2.6 (5.8)	ND	ND - 25.0
Haemophilus	1/52	0.1 (0.5)	ND	ND - 3.6
Eikenella	1/52	0.05 (0.4)	ND	ND - 2.8
Unidentified	6/52	0.7 (2.0)	ND	ND - 8.3

SECTION - SUCROSE PLAQUE

	F	Mean (SD)	Median	Range
+ve cocci	52/52	42.3 (23.8)	35.3	3.6 - 87.1
S. mutans	34/52	2.4 (6.6)	0.1	ND - 32.3
S. sanguis	31/52	10.2 (14.3)	4.4	ND - 61.3
S. oralis	35/52	19.7 (21.5)	13.8	ND - 69.6
S. salivarius	12/52	6.3 (16.9)	ND	ND - 77.1
S. milleri	4/52	0.6 (2.7)	ND	ND - 18.5
Other Strep.	7/52	1.6 (5.6)	ND	ND - 29.6
ANO_2 Strep.	9/52	1.4 (3.7)	ND	ND - 15.9
Micrococcus/Staph.	2/52	0.2 (0.9)	ND	ND - 4.8
-ve cocci	37/52	8.0 (9.2)	4.8	ND - 41.7
Veillonella	37/52	7.7 (9.0)	4.4	ND - 41.7
Neisseria	5/52	0.4 (1.2)	ND	ND - 4.8
+ve bacilli	52/52	41.3 (25.4)	42.4	3.0 - 93.0
A. odontolyticus	42/52	18.5 (18.9)	12.9	ND - 79.3
A. v/n - cat. +ve	25/52	7.2 (10.0)	ND	ND - 38.7
A. v/n - cat. -ve	32/52	9.2 (10.3)	6.6	ND - 31.6
Other Actinomyces	1/52	0.7 (5.1)	ND	ND - 37.0
Lactobacillus	35/52	2.7 (7.5)	0.1	ND - 41.4
Bifidobacterium	9/52	2.1 (6.2)	ND	ND - 29.0
Propionibacterium	1/52	0.1 (0.4)	ND	ND - 3.2
Arachnia	0/52	ND	ND	ND
Eubacterium	6/52	1.0 (4.5)	ND	ND - 31.6
Unidentified	3/52	0.3 (1.6)	ND	ND - 10.5
-ve bacilli	35/52	8.0 (9.1)	5.6	ND - 40.8
Bacteroides	19/52	3.9 (6.5)	ND	ND - 24.3
Fusobacterium	7/52	0.5 (1.4)	ND	ND - 5.9
Capnocytophaga	17/52	2.4 (5.5)	ND	ND - 29.2
Haemophilus	1/52	0.1 (0.4)	ND	ND - 3.1
Eikenella	3/52	0.3 (1.1)	ND	ND - 6.7
Unidentified	6/52	1.0 (3.2)	ND	ND - 13.7

Table 4.11. cont.

SECTION - SPM

	F	Mean (SD)	Median	Range
+ve cocci	39/40	42.9 (25.2)	45.6	ND - 91.3
S. mutans	35/40	21.0 (29.0)	0.1	ND - 88.3
S. sanguis	21/40	6.9 (10.4)	2.2	ND - 35.3
S. oralis	28/40	12.7 (15.0)	9.2	ND - 59.1
S. salivarius	5/40	0.6 (1.9)	ND	ND - 8.7
S. milleri	2/40	0.1 (0.6)	ND	ND - 2.9
Other Strep.	2/40	0.2 (0.8)	ND	ND - 3.7
ANO_2 Strep.	8/40	1.2 (3.2)	ND	ND - 15.4
Micrococcus/Staph.	2/40	0.2 (1.0)	ND	ND - 4.5
-ve cocci	32/40	8.9 (10.1)	5.0	ND - 39.3
Veillonella	31/40	8.7 (9.9)	5.0	ND - 39.3
Neisseria	2/40	0.2 (1.0)	ND	ND - 5.3
+ve bacilli	40/40	44.7 (25.3)	40.4	0.5 - 96.6
A. odontolyticus	32/40	13.5 (12.4)	12.2	ND - 48.3
A. v/n cat. +ve	19/40	10.5 (17.6)	ND	ND - 73.7
A. v/n cat. -ve	22/40	7.6 (10.9)	2.5	ND - 48.3
Other Actinomyces	5/40	2.2 (9.8)	ND	ND - 60.0
Lactobacillus	35/40	7.7 (13.8)	0.2	ND - 49.0
Bifidobacterium	7/40	0.9 (2.2)	ND	ND - 8.6
Propionibacterium	1/40	0.1 (0.6)	ND	ND - 3.7
Arachnia	1/40	0.1 (0.5)	ND	ND - 3.4
Eubacterium	4/40	2.1 (7.9)	ND	ND - 38.1
Unidentified	2/40	0.1 (0.6)	ND	ND - 3.7
-ve bacilli	19/40	3.4 (4.4)	ND	ND - 12.5
Bacteroides	14/40	2.0 (3.6)	ND	ND - 11.8
Fusobacterium	2/40	0.2 (0.7)	ND	ND - 3.6
Capnocytophaga	7/40	1.0 (2.8)	ND	ND - 11.1
Haemophilus	1/40	0.1 (0.5)	ND	ND - 3.1
Eikenella	1/40	0.01 (0.1)	ND	ND - 0.5
Unidentified	1/40	0.05 (0.3)	ND	ND - 2.1

Table 4.11. cont.

Table 4.12. % predominant cultivable plaque microflora isolated from enamel slabs, under Protocol I (NP), for Subjects A - G.

SLAB - NORMAL PLAQUE
SUBJECT A

	F	Mean (SD)	Median	Range
+ve cocci	7/7	30.2 (25.2)	18.2	6.9 - 68.0
S. mutans	0/7	ND	ND	ND
S. sanguis	7/7	20.7 (21.8)	15.2	4.5 - 68.0
S. oralis	3/7	7.3 (15.4)	ND	ND - 41.5
-ve cocci	7/7	14.1 (17.0)	4.3	2.4 - 41.4
Veillonella	7/7	14.1 (17.0)	4.3	2.4 - 41.4
+ve bacilli	7/7	51.3 (23.3)	45.4	16.0 - 83.7
Actinomyces	7/7	49.9 (23.7)	44.8	16.0 - 83.7
Lactobacillus	2/7	0.2 (0.4)	ND	ND - 1.1
-ve bacilli	4/7	4.3 (4.6)	4.3	ND - 12.0
Bacteroides	2/7	1.2 (2.0)	ND	ND - 4.3

SLAB - NORMAL PLAQUE
SUBJECT B

	F	Mean (SD)	Median	Range
+ve cocci	8/8	28.6 (14.1)	31.6	3.4 - 47.6
S. mutans	4/8	1.1 (2.1)	0.05	ND - 5.4
S. sanguis	6/8	11.0 (9.8)	11.6	ND - 28.6
S. oralis	7/8	13.6 (8.9)	15.6	ND - 24.2
-ve cocci	6/8	11.0 (10.4)	10.0	ND - 33.3
Veillonella	6/8	10.4 (10.4)	9.2	ND - 33.3
+ve bacilli	8/8	58.7 (21.8)	54.4	19.0 - 86.5
Actinomyces	8/8	55.0 (19.6)	52.7	19.0 - 75.8
Lactobacillus	4/8	1.4 (3.8)	0.05	ND - 10.8
-ve bacilli	3/8	1.8 (2.6)	ND	ND - 6.7
Bacteroides	3/8	1.8 (2.6)	ND	ND - 6.7

SLAB - NORMAL PLAQUE
SUBJECT C

	F	Mean (SD)	Median	Range
+ve cocci	7/7	47.0 (10.0)	45.0	36.4 - 64.3
S. mutans	5/7	0.07 (0.05)	0.1	ND - 0.1
S. sanguis	6/7	18.8 (14.2)	18.2	ND - 35.7
S. oralis	6/7	21.4 (16.5)	21.0	ND - 48.1
-ve cocci	6/7	13.2 (11.4)	13.6	ND - 35.3
Veillonella	6/7	11.7 (11.4)	9.1	ND - 35.3
+ve bacilli	7/7	32.0 (12.3)	33.3	17.6 - 50.0
Actinomyces	7/7	30.6 (10.7)	33.3	17.6 - 45.4
Lactobacillus	7/7	0.1 (0.0)	0.1	0.1
-ve bacilli	6/7	7.8 (4.5)	7.4	ND - 14.3
Bacteroides	3/7	2.3 (2.9)	ND	ND - 5.9

SLAB - NORMAL PLAQUE
SUBJECT D

	F	Mean (SD)	Median	Range
+ve cocci	7/7	22.0 (17.3)	27.8	0.1 - 42.8
S. mutans	6/7	0.1 (0.04)	0.1	ND - 0.1
S. sanguis	4/7	8.2 (7.8)	12.9	ND - 16.7
S. oralis	5/7	11.2 (9.5)	11.1	ND - 25.0
-ve cocci	4/7	2.5 (2.4)	3.2	ND - 5.6
Veillonella	4/7	2.5 (2.4)	3.2	ND - 5.6
+ve bacilli	7/7	71.9 (16.0)	61.3	57.1 - 95.6
Actinomyces	7/7	68.9 (14.4)	61.1	57.1 - 95.6
Lactobacillus	5/7	1.7 (2.1)	0.1	ND - 4.3
-ve bacilli	5/7	3.6 (3.1)	3.4	ND - 8.7
Bacteroides	3/7	2.4 (2.4)	3.2	ND - 5.6

Table 4.12. cont.

SLAB - NORMAL PLAQUE
SUBJECT E

	F	Mean (SD)	Median	Range
+ve cocci	8/8	27.6 (21.5)	28.0	0.1 - 54.8
S. mutans	8/8	13.9 (15.2)	9.6	0.1 - 38.7
S. sanguis	3/8	2.9 (4.5)	ND	ND - 11.1
S. oralis	6/8	5.7 (5.0)	5.3	ND - 15.3
-ve cocci	6/8	5.9 (4.5)	6.5	ND - 12.1
Veillonella	5/8	4.4 (4.1)	4.6	ND - 9.7
+ve bacilli	8/8	64.0 (27.1)	61.6	35.5 - 94.7
Actinomyces	8/8	59.4 (26.3)	57.6	32.3 - 89.4
Lactobacillus	8/8	1.5 (1.9)	0.1	0.1 - 4.2
-ve bacilli	3/8	2.5 (3.7)	ND	ND - 8.3
Bacteroides	3/8	2.0 (3.0)	ND	ND - 8.0

SLAB - NORMAL PLAQUE
SUBJECT F

	F	Mean (SD)	Median	Range
+ve cocci	8/8	22.3 (8.4)	21.4	7.1 - 36.0
S. mutans	3/8	0.04 (0.05)	ND	ND - 0.1
S. sanguis	7/8	7.0 (5.0)	6.1	ND - 13.0
S. oralis	8/8	12.5 (6.3)	12.0	3.6 - 25.0
-ve cocci	7/8	6.2 (3.4)	7.0	ND - 10.7
Veillonella	6/8	4.3 (3.6)	4.1	ND - 10.7
+ve bacilli	8/8	51.9 (19.8)	55.7	8.0 - 71.4
Actinomyces	8/8	45.2 (16.9)	49.0	8.0 - 64.2
Lactobacillus	4/8	4.7 (7.4)	1.8	ND - 21.6
-ve bacilli	8/8	19.6 (15.0)	16.4	4.3 - 52.0
Bacteroides	8/8	12.5 (10.3)	9.5	4.0 - 36.0

Table 4.12. cont.

SLAB - NORMAL PLAQUE
SUBJECT G

	F	Mean (SD)	Median	Range
+ve cocci	4/4	74.3 (4.1)	74.6	69.2 - 78.8
S. mutans	0/4	ND	ND	ND
S. sanguis	4/4	32.8 (15.6)	31.0	19.2 - 50.0
S. oralis	4/4	24.0 (13.6)	22.1	12.0 - 40.0
-ve cocci	4/4	8.3 (3.3)	8.8	4.0 - 11.5
Veillonella	4/4	8.3 (3.3)	8.8	4.0 - 11.5
+ve bacilli	4/4	8.8 (1.3)	8.8	7.7 - 10.0
Actinomyces	4/4	8.8 (1.3)	8.8	7.7 - 10.0
Lactobacillus	4/4	0.1 (0.0)	0.1	0.1
-ve bacilli	4/4	8.5 (5.7)	8.4	1.9 - 15.4
Bacteroides	2/4	1.8 (2.1)	1.6	ND - 3.8

Table 4.12. cont.

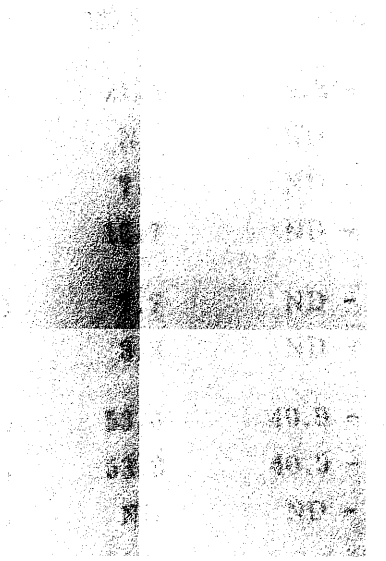


Table 4.13. % predominant cultivable plaque microflora isolated from enamel slabs, under Protocol II (SP), for Subjects A - G.

SLAB - SUCROSE PLAQUE
SUBJECT A

	F	Mean (SD)	Median	Range
+ve cocci	8/8	50.2 (11.0)	52.2	33.3 - 65.4
S. mutans	3/8	0.04 (0.05)	ND	ND - 0.1
S. sanguis	7/8	13.6 (11.5)	10.1	ND - 29.2
S. oralis	8/8	25.4 (14.4)	29.8	4.8 - 44.4
-ve cocci	6/8	4.6 (3.6)	4.4	ND - 9.5
Veillonella	6/8	4.6 (3.6)	4.4	ND - 9.5
+ve bacilli	8/8	34.4 (10.1)	34.4	22.2 - 47.6
Actinomyces	8/8	29.9 (8.4)	27.9	19.2 - 42.8
Lactobacillus	5/8	1.4 (2.4)	0.1	ND - 5.6
-ve bacilli	7/8	10.4 (5.3)	10.1	ND - 16.7
Bacteroides	6/8	6.4 (6.6)	4.2	ND - 16.7

SLAB - SUCROSE PLAQUE
SUBJECT B

	F	Mean (SD)	Median	Range
+ve cocci	7/7	21.8 (9.6)	21.4	8.3 - 33.3
S. mutans	2/7	0.03 (0.05)	ND	ND - 0.1
S. sanguis	4/7	7.8 (8.5)	7.7	ND - 20.8
S. oralis	6/7	14.0 (8.2)	16.7	ND - 23.1
-ve cocci	5/7	7.3 (5.7)	7.7	ND - 14.3
Veillonella	5/7	5.6 (5.2)	3.8	ND - 14.3
+ve bacilli	7/7	55.8 (11.9)	53.6	40.9 - 75.0
Actinomyces	7/7	54.1 (12.8)	53.6	40.9 - 75.0
Lactobacillus	2/7	0.03 (0.05)	ND	ND - 0.1
-ve bacilli	7/7	14.0 (6.0)	12.5	7.1 - 20.8
Bacteroides	7/7	11.6 (6.4)	8.3	7.1 - 20.8

SLAB - SUCROSE PLAQUE
SUBJECT C

	F	Mean (SD)	Median	Range
+ve cocci	4/7	44.5 (13.5)	44.0	26.7 - 64.3
S. mutans	4/7	0.1 (0.05)	0.1	ND - 0.1
S. sanguis	6/7	16.2 (10.1)	15.4	ND - 28.6
S. oralis	7/7	26.6 (12.2)	27.3	11.5 - 43.4
-ve cocci	7/7	7.5 (3.9)	6.7	3.8 - 14.3
Veillonella	7/7	7.5 (3.9)	6.7	3.8 - 14.3
+ve bacilli	7/7	36.7 (17.4)	40.0	3.6 - 56.7
Actinomyces	7/7	34.9 (16.7)	36.3	3.6 - 56.7
Lactobacillus	4/7	0.1 (0.05)	0.1	ND - 0.1
-ve bacilli	7/7	12.5 (4.5)	13.0	4.5 - 17.8
Bacteroides	3/7	2.4 (3.1)	ND	ND - 7.7

SLAB - SUCROSE PLAQUE
SUBJECT D

	F	Mean (SD)	Median	Range
+ve cocci	8/8	43.9 (22.6)	34.0	14.3 - 75.9
S. mutans	8/8	0.1 (0.0)	0.1	0.1
S. sanguis	8/8	19.0 (10.4)	19.0	4.0 - 37.9
S. oralis	8/8	22.5 (14.6)	19.8	3.6 - 43.2
-ve cocci	4/8	3.0 (3.4)	2.1	ND - 8.0
Veillonella	4/8	3.0 (3.4)	2.1	ND - 8.0
+ve bacilli	8/8	47.8 (21.0)	53.0	13.8 - 75.0
Actinomyces	8/8	46.8 (20.0)	51.0	13.8 - 71.4
Lactobacillus	5/8	0.6 (1.5)	0.1	ND - 4.2
-ve bacilli	6/8	5.4 (4.6)	5.0	ND - 13.0
Bacteroides	3/8	1.9 (3.1)	ND	ND - 8.7

Table 4.13. cont.

SLAB - SUCROSE PLAQUE
SUBJECT E

	F	Mean (SD)	Median	Range
+ve cocci	8/8	33.4 (13.9)	31.3	16.0 - 56.2
S. mutans	7/8	20.6 (23.6)	10.2	ND - 56.2
S. sanguis	4/8	8.3 (9.4)	4.6	ND - 20.0
S. oralis	3/8	4.1 (5.9)	ND	ND - 13.6
-ve cocci	5/8	3.9 (3.7)	4.0	ND - 9.1
Veillonella	4/8	3.0 (3.5)	2.0	ND - 9.1
+ve bacilli	8/8	56.8 (16.2)	54.1	37.5 - 84.0
Actinomyces	8/8	53.4 (18.4)	51.1	31.2 - 84.0
Lactobacillus	8/8	2.2 (2.9)	0.6	0.1 - 8.0
-ve bacilli	4/8	5.8 (8.2)	2.2	ND - 23.1
Bacteroides	4/8	3.9 (4.6)	2.2	ND - 11.5

SLAB - SUCROSE PLAQUE
SUBJECT F

	F	Mean (SD)	Median	Range
+ve cocci	8/8	16.6 (7.7)	15.4	8.0 - 33.0
S. mutans	5/8	0.1 (0.05)	0.1	ND - 0.1
S. sanguis	4/8	4.6 (6.0)	1.8	ND - 16.7
S. oralis	8/8	10.1 (3.5)	8.3	7.1 - 16.7
-ve cocci	6/8	7.2 (5.5)	8.0	ND - 13.6
Veillonella	6/8	6.2 (4.4)	7.8	ND - 12.0
+ve bacilli	8/8	50.7 (13.4)	49.2	32.0 - 69.6
Actinomyces	8/8	43.3 (8.8)	41.8	32.0 - 56.7
Lactobacillus	3/8	0.04 (0.05)	ND	ND - 0.1
-ve bacilli	8/8	25.0 (19.1)	19.1	4.3 - 60.0
Bacteroides	6/8	13.5 (18.3)	8.2	ND - 56.0

Table 4.13. cont.

SLAB - SUCROSE PLAQUE
SUBJECT G

	F	Mean (SD)	Median	Range
+ve cocci	4/4	76.4 (7.3)	74.5	70.0 - 86.7
S. mutans	0/4	ND	ND	ND
S. sanguis	2/4	4.7 (5.8)	3.4	ND - 12.0
S. oralis	4/4	32.7 (19.4)	32.8	13.3 - 52.0
-ve cocci	4/4	20.9 (7.7)	20.2	13.3 - 30.0
Veillonella	4/4	20.9 (7.7)	20.2	13.3 - 30.0
+ve bacilli	4/4	2.0 (4.0)	0.1	0.1 - 8.0
Actinomyces	1/4	2.0 (4.0)	ND	ND - 8.0
Lactobacillus	4/4	0.1 (0.0)	0.1	0.1
-ve bacilli	1/4	0.7 (1.4)	ND	ND - 2.7
Bacteroides	0/4	ND	ND	ND

Table 4.13. cont.

Table 4.14. % predominant cultivable plaque microflora isolated from enamel slabs, under Protocol III (SPM), for Subjects A - E.

SLAB - SPM
SUBJECT A

	F	Mean (SD)	Median	Range
+ve cocci	8/8	64.3 (19.6)	68.6	19.2 - 83.9
S. mutans	8/8	47.8 (19.5)	50.8	17.3 - 77.4
S. sanguis	5/8	3.5 (4.2)	2.4	ND - 11.4
S. oralis	6/8	10.2 (12.7)	3.2	ND - 29.5
-ve cocci	7/8	5.8 (3.7)	5.4	ND - 11.4
Veillonella	7/8	5.8 (3.7)	5.4	ND - 11.4
+ve bacilli	8/8	26.0 (22.3)	20.6	3.2 - 76.9
Actinomyces	8/8	22.8 (17.5)	19.0	3.2 - 61.5
Lactobacillus	7/8	1.3 (3.5)	0.1	ND - 9.9
-ve bacilli	4/8	4.0 (4.9)	1.6	ND - 12.0
Bacteroides	4/8	1.7 (1.9)	1.6	ND - 4.0

SLAB - SPM
SUBJECT B

	F	Mean (SD)	Median	Range
+ve cocci	8/8	23.8 (9.5)	26.0	8.0 - 35.7
S. mutans	6/8	1.0 (2.6)	0.1	ND - 7.3
S. sanguis	4/8	5.4 (7.0)	2.4	ND - 18.5
S. oralis	6/8	15.8 (13.1)	15.4	ND - 35.7
-ve cocci	5/8	11.4 (13.6)	5.8	ND - 36.0
Veillonella	5/8	11.4 (13.6)	5.8	ND - 36.0
+ve bacilli	8/8	58.9 (8.5)	56.9	44.4 - 73.2
Actinomyces	8/8	55.1 (10.0)	56.6	37.0 - 66.7
Lactobacillus	6/8	2.2 (4.0)	0.1	ND - 9.8
-ve bacilli	5/8	5.9 (5.4)	6.6	ND - 13.0
Bacteroides	5/8	5.9 (5.4)	6.6	ND - 13.0

SLAB - SPM
SUBJECT C

	F	Mean (SD)	Median	Range
+ve cocci	7/7	13.9 (8.2)	11.8	6.2 - 26.9
S. mutans	7/7	1.2 (1.9)	0.1	0.1 - 4.3
S. sanguis	5/7	5.7 (5.5)	4.3	ND - 14.3
S. oralis	4/7	4.6 (4.8)	4.8	ND - 11.8
-ve cocci	6/7	8.8 (5.0)	11.1	ND - 13.0
Veillonella	6/7	8.2 (5.2)	11.1	ND - 13.0
+ve bacilli	7/7	72.1 (8.3)	66.7	64.7 - 81.5
Actinomyces	7/7	63.1 (15.2)	64.7	37.0 - 81.2
Lactobacillus	7/7	9.0 (16.2)	0.1	0.1 - 44.4
-ve bacilli	3/7	5.1 (8.7)	ND	ND - 23.5
Bacteroides	2/7	4.6 (8.9)	ND	ND - 23.5

SLAB - SPM
SUBJECT D

	F	Mean (SD)	Median	Range
+ve cocci	8/8	28.4 (11.6)	25.6	12.8 - 52.2
S. mutans	6/8	1.3 (2.2)	0.1	ND - 5.0
S. sanguis	4/8	5.2 (7.8)	2.4	ND - 22.2
S. oralis	7/8	20.2 (14.2)	19.1	ND - 47.8
-ve cocci	8/8	8.5 (3.6)	9.5	4.2 - 13.0
Veillonella	8/8	7.3 (2.9)	7.2	4.2 - 10.9
+ve bacilli	8/8	52.2 (13.6)	54.8	26.1 - 72.3
Actinomyces	8/8	49.4 (13.6)	52.3	23.9 - 72.3
Lactobacillus	7/8	1.6 (2.2)	0.1	ND - 5.0
-ve bacilli	7/8	10.9 (8.8)	9.5	ND - 31.1
Bacteroides	6/8	3.5 (2.4)	4.6	ND - 6.4

Table 4.14. cont.

SLAB - SPM
SUBJECT E

	F	Mean (SD)	Median	Range
+ve cocci	8/8	59.1 (15.4)	63.0	25.9 - 74.0
S. mutans	8/8	52.3 (13.8)	54.2	25.9 - 74.0
S. sanguis	2/8	1.6 (3.2)	ND	ND - 8.3
S. oralis	6/8	4.2 (3.1)	4.2	ND - 8.0
-ve cocci	5/8	5.0 (5.6)	3.8	ND - 14.8
Veillonella	5/8	5.0 (5.6)	3.8	ND - 14.8
+ve bacilli	8/8	33.8 (16.3)	32.0	14.8 - 66.7
Actinomyces	8/8	30.2 (19.0)	28.7	3.7 - 66.7
Lactobacillus	7/8	3.6 (3.6)	2.8	ND - 11.1
-ve bacilli	3/8	2.1 (3.2)	ND	ND - 7.4
Bacteroides	3/8	1.6 (2.5)	ND	ND - 6.7

Table 4.14. cont.

Table 4.15. % predominant cultivable plaque microflora isolated from enamel slabs, under each treatment condition, for five Subjects, A - E, combined.

SLAB - NORMAL PLAQUE
SUBJECTS A - E

	F	Mean (SD)	Median	Range
+ve cocci	37/37	30.9 (19.2)	34.8	0.1 - 68.0
S. mutans	23/37	3.3 (8.8)	0.1	ND - 38.7
S. sanguis	26/37	12.0 (13.8)	10.0	ND - 68.0
S. oralis	27/37	11.7 (12.3)	8.7	ND - 48.1
-ve cocci	29/37	9.3 (10.8)	5.6	ND - 41.4
Veillonella	28/37	8.6 (10.7)	5.3	ND - 41.4
+ve bacilli	37/37	55.9 (24.0)	52.0	16.0 - 95.6
Actinomyces	37/37	53.0 (22.7)	52.0	16.0 - 95.6
Lactobacillus	26/37	1.0 (2.2)	0.1	ND - 10.8
-ve bacilli	21/37	3.9 (4.1)	3.4	ND - 14.3
Bacteroides	15/37	1.9 (2.5)	ND	ND - 8.0

SLAB - SUCROSE PLAQUE
SUBJECTS A - E

	F	Mean (SD)	Median	Range
+ve cocci	38/38	39.0 (17.4)	34.7	8.3 - 75.9
S. mutans	24/38	4.4 (13.3)	0.1	ND - 56.2
S. sanguis	29/38	12.8 (10.5)	11.8	ND - 37.9
S. oralis	32/38	18.4 (14.0)	15.8	ND - 44.4
-ve cocci	27/38	5.1 (4.3)	4.4	ND - 14.3
Veillonella	26/38	4.6 (4.1)	4.2	ND - 14.3
+ve bacilli	38/38	46.3 (17.7)	46.7	3.6 - 84.0
Actinomyces	38/38	43.8 (18.0)	42.6	3.6 - 84.0
Lactobacillus	24/38	0.9 (2.0)	0.1	ND - 8.0
-ve bacilli	31/38	9.4 (6.6)	9.8	ND - 23.1
Bacteroides	23/38	5.1 (5.8)	4.0	ND - 20.8

SLAB - SPM
SUBJECTS A - E

	F	Mean (SD)	Median	Range
+ve cocci	39/39	38.5 (24.0)	27.5	6.2 - 83.9
S. mutans	35/39	21.2 (26.5)	4.3	ND - 77.4
S. sanguis	20/39	4.3 (5.7)	1.9	ND - 22.2
S. oralis	29/39	11.2 (12.0)	7.4	ND - 47.8
-ve cocci	31/39	7.9 (7.4)	6.7	ND - 36.0
Veillonella	31/39	7.5 (7.3)	5.0	ND - 36.0
+ve bacilli	39/39	48.0 (21.9)	56.0	3.2 - 81.5
Actinomyces	39/39	43.6 (21.1)	45.0	3.2 - 81.2
Lactobacillus	34/39	3.4 (7.6)	0.1	ND - 44.4
-ve bacilli	22/39	5.6 (6.9)	3.8	ND - 31.1
Bacteroides	20/39	3.4 (4.9)	2.4	ND - 23.5

Table 4.15. cont.

Table 4.16. % total cultivable plaque microflora isolated from enamel slabs, under each treatment condition, for all Subjects, combined.

SLAB - NORMAL PLAQUE

	F	Mean (SD)	Median	Range
+ve cocci	49/49	33.0 (21.3)	33.3	0.1 - 78.8
S. mutans	26/49	2.5 (7.8)	0.1	ND - 38.7
S. sanguis	37/49	12.9 (14.2)	10.0	ND - 68.0
S. oralis	39/49	12.9 (11.9)	9.1	ND - 48.1
S. salivarius	14/49	2.6 (5.4)	ND	ND - 20.0
S. milleri	14/49	0.8 (4.2)	ND	ND - 28.6
Other Strep.	1/49	0.4 (2.9)	ND	ND - 20.0
ANO_2 Strep.	10/49	0.9 (2.1)	ND	ND - 9.7
Micrococcus/Staph.	4/49	0.2 (0.7)	ND	ND - 3.0
-ve cocci	40/49	8.7 (9.5)	7.0	ND - 41.4
Veillonella	38/49	7.8 (9.6)	5.0	ND - 41.4
Neisseria	9/49	0.9 (1.9)	ND	ND - 7.7
+ve bacilli	49/49	51.4 (25.6)	50.0	7.7 - 95.6
A. odontolyticus	45/49	16.3 (13.7)	13.6	ND - 58.6
A. v/n - cat. +ve	37/49	18.1 (20.4)	12.5	ND - 82.6
A. v/n - cat. -ve	38/49	13.5 (14.8)	8.7	ND - 64.0
Other Actinomyces	2/49	0.3 (1.4)	ND	ND - 9.1
Lactobacillus	34/49	1.5 (3.7)	0.1	ND - 21.6
Bifidobacterium	10/49	0.8 (1.6)	ND	ND - 4.5
Propionibacterium	1/49	0.1 (0.5)	ND	ND - 3.4
Arachnia	0/49	ND	ND	ND
Eubacterium	8/49	0.7 (1.6)	ND	ND - 5.3
Unidentified	1/49	0.2 (1.3)	ND	ND - 9.1
-ve bacilli	33/49	6.8 (9.0)	4.3	ND - 52.0
Bacteroides	24/49	3.6 (6.0)	ND	ND - 36.0
Fusobacterium	3/49	0.4 (1.6)	ND	ND - 8.7
Capnocytophaga	11/49	1.6 (3.5)	ND	ND - 16.0
Haemophilus	2/49	0.3 (1.4)	ND	ND - 8.7
Eikenella	5/49	0.7 (2.5)	ND	ND - 14.3
Unidentified	6/49	0.4 (1.2)	ND	ND - 5.3

SLAB - SUCROSE PLAQUE

	F	Mean (SD)	Median	Range
+ve cocci	50/50	38.4 (20.9)	33.3	8.0 - 86.7
S. mutans	29/50	3.3 (11.7)	0.1	ND - 56.2
S. sanguis	35/50	11.0 (10.2)	9.1	ND - 37.9
S. oralis	44/50	18.2 (14.2)	13.6	ND - 52.0
S. salivarius	13/50	4.9 (12.5)	ND	ND - 56.7
S. milleri	1/50	0.1 (0.4)	ND	ND - 2.8
Other Strep.	6/50	0.5 (1.3)	ND	ND - 4.5
ANO_2 Strep.	5/50	0.5 (1.9)	ND	ND - 10.0
Micrococcus/Staph.	0/50	ND	ND	ND
-ve cocci	37/50	6.7 (6.3)	5.9	ND - 30.0
Veillonella	36/50	6.2 (6.2)	4.4	ND - 30.0
Neisseria	7/50	0.6 (1.4)	ND	ND - 4.5
+ve bacilli	50/50	43.4 (20.4)	43.6	0.1 - 84.0
A. odontolyticus	36/50	11.5 (11.8)	8.0	ND - 46.7
A. v/n - cat. +ve	37/50	15.2 (16.8)	12.8	ND - 84.0
A. v/n - cat. -ve	38/50	13.4 (15.8)	8.3	ND - 75.0
Other Actinomyces	4/50	0.3 (1.1)	ND	ND - 4.3
Lactobacillus	31/50	0.7 (1.8)	0.1	ND - 8.0
Bifidobacterium	10/50	1.4 (4.4)	ND	ND - 29.6
Propionibacterium	4/50	0.4 (1.5)	ND	ND - 8.0
Arachnia	0/50	ND	ND	ND
Eubacterium	5/50	0.5 (1.9)	ND	ND - 11.1
Unidentified	2/50	0.1 (0.9)	ND	ND - 6.2
-ve bacilli	40/50	11.2 (11.3)	10.2	ND - 60.0
Bacteroides	29/50	6.0 (9.3)	3.7	ND - 56.0
Fusobacterium	9/50	0.8 (1.8)	ND	ND - 7.7
Capnocytophaga	17/50	2.3 (3.9)	ND	ND - 17.8
Haemophilus	2/50	0.1 (0.7)	ND	ND - 3.8
Eikenella	7/50	0.5 (1.3)	ND	ND - 4.5
Unidentified	2/50	0.1 (0.9)	ND	ND - 0.9

Table 4.16. cont.

SLAB - SPM

	F	Mean (SD)	Median	Range
+ve cocci	39/39	38.5 (24.0)	27.5	6.2 - 83.9
S. mutans	35/39	21.2 (26.5)	4.3	ND - 77.4
S. sanguis	20/39	4.3 (5.7)	1.9	ND - 22.2
S. oralis	29/39	11.2 (12.0)	7.4	ND - 47.8
S. salivarius	5/39	0.7 (2.0)	ND	ND - 9.8
S. milleri	0/39	ND	ND	ND
Other Strep.	2/39	0.2 (1.0)	ND	ND - 4.3
ANO_2 Strep.	8/39	1.0 (2.3)	ND	ND - 11.7
Micrococcus/Staph.	0/39	ND	ND	ND
-ve cocci	31/39	7.9 (7.4)	6.7	ND - 36.0
Veillonella	31/39	7.5 (7.3)	5.0	ND - 36.0
Neisseria	4/39	0.4 (1.2)	ND	ND - 5.0
+ve bacilli	39/39	48.0 (21.9)	56.0	3.2 - 81.5
A. odontolyticus	37/39	14.5 (12.0)	11.4	ND - 48.8
A. v/n - cat. +ve	31/39	18.6 (18.4)	14.8	ND - 72.0
A. v/n - cat. -ve	25/39	11.1 (13.5)	6.7	ND - 51.2
Other Actinomyces	2/39	0.2 (0.8)	ND	ND - 4.8
Lactobacillus	34/39	3.4 (7.6)	0.1	ND - 44.4
Bifidobacterium	2/39	0.6 (2.9)	ND	ND - 15.4
Propionibacterium	0/39	ND	ND	ND
Arachnia	0/39	ND	ND	ND
Eubacterium	3/39	0.3 (1.2)	ND	ND - 5.0
Unidentified	0/39	ND	ND	ND
-ve bacilli	22/39	5.6 (6.9)	3.8	ND - 31.1
Bacteroides	20/39	3.4 (4.9)	2.4	ND - 23.5
Fusobacterium	1/39	0.1 (0.6)	ND	ND - 3.7
Capnocytophaga	10/39	1.2 (2.3)	ND	ND - 8.0
Haemophilus	1/39	0.1 (0.7)	ND	ND - 4.3
Eikenella	0/39	ND	ND	ND
Unidentified	2/39	0.7 (3.9)	ND	ND - 24.4

Table 4.16. cont.

	Side	Position	Run	Subject	Treatment
+ve cocci	NS	NS	**	**	NS
S. mutans	NS	NS	NS	***	***
S. sanguis	NS	NS	*	***	NS
S. oralis	NS	NS	***	***	**
-ve cocci	NS	NS	NS	NS	NS
Veillonella	NS	NS	NS	*	NS
+ve bacilli	NS	NS	***	***	NS
Actinomyces	NS	NS	NS	***	NS
Lactobacillus	NS	NS	*	***	*
-ve bacilli	NS	NS	*	**	*
Bacteroides	NS	NS	NS	*	NS

NS = Not significant ; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Table 4.17. Statistical analysis of effect of side, position, run, subject and treatment conditions on predominant cultivable plaque microflora isolated from enamel sections.

	Side	Position	Run	Subject	Treatment
+ve cocci	NS	NS	NS	***	NS
S. mutans	NS	NS	NS	***	***
S. sanguis	*	NS	NS	**	**
S. oralis	NS	NS	NS	***	*
-ve cocci	NS	NS	NS	**	NS
Veillonella	NS	NS	NS	**	NS
+ve bacilli	NS	NS	NS	***	NS
Actinomyces	NS	NS	NS	***	NS
Lactobacillus	NS	NS	NS	NS	NS
-ve bacilli	NS	NS	**	***	**
Bacteroides	NS	NS	*	***	*

NS = Not significant ; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Table 4.18. Statistical analysis of effect of side, position, run, subject and treatment conditions on predominant cultivable plaque microflora isolated from enamel slabs.

Table 4.19. Total microbial counts (log_{10} cfu/mm^2 enamel surface), and absolute counts of predominant cultivable plaque microflora isolated from enamel slabs, under each treatment condition, for Subjects A - G.

SUBJECT A

	NP, n=7 Mean (SD)	SP, n=8 Mean (SD)	SPM, n=8 Mean (SD)
+ve cocci	6.00 (0.74)	6.07 (0.49)	6.92 (0.41)
S. mutans	0.38 (0.63)	0.86 (1.36)	6.80 (0.42)
S. sanguis	5.83 (0.61)	5.37 (0.36)	5.60 (1.46)
S. oralis	5.06 (1.06)	5.71 (0.66)	5.85 (0.68)
-ve cocci	5.61 (0.27)	4.99 (0.57)	5.88 (0.33)
Veillonella	5.61 (0.27)	4.99 (0.57)	5.88 (0.33)
+ve bacilli	6.30 (0.57)	5.91 (0.40)	6.44 (0.26)
Actinomyces	6.28 (0.58)	5.85 (0.42)	6.41 (0.22)
Lactobacillus	0.97 (1.88)	2.08 (2.24)	2.36 (2.01)
-ve bacilli	5.17 (0.62)	5.36 (0.69)	5.60 (0.62)
Bacteroides	4.84 (0.59)	5.06 (0.68)	5.47 (0.47)
Total	6.63 (0.50)	6.38 (0.44)	7.17 (0.23)

SUBJECT B

	NP, n=8 Mean (SD)	SP, n=7 Mean (SD)	SPM, n=8 Mean (SD)
+ve cocci	7.10 (0.38)	6.62 (0.42)	6.98 (0.54)
S. mutans	2.8 (2.97)	1.44 (1.92)	2.96 (1.78)
S. sanguis	6.48 (0.38)	5.94 (0.82)	6.16 (1.03)
S. oralis	6.72 (0.49)	6.34 (0.46)	6.61 (1.03)
-ve cocci	6.46 (0.50)	6.05 (0.61)	6.35 (1.20)
Veillonella	6.44 (0.50)	5.95 (0.60)	6.35 (1.20)
+ve bacilli	7.36 (0.60)	7.04 (0.23)	7.40 (0.59)
Actinomyces	7.35 (0.58)	7.02 (0.23)	7.37 (0.58)
Lactobacillus	1.44 (2.42)	0.70 (0.65)	2.46 (2.73)
-ve bacilli	6.00 (0.32)	6.45 (0.41)	6.24 (0.65)
Bacteroides	5.97 (0.32)	6.36 (0.35)	6.24 (0.65)
Total	7.64 (0.48)	7.33 (0.27)	7.62 (0.63)

SUBJECT C

	NP, n=7 Mean (SD)	SP, n=7 Mean (SD)	SPM, n=7 Mean (SD)
+ve cocci	6.81 (0.67)	6.55 (0.20)	6.65 (0.37)
S. mutans	2.36 (2.01)	2.16 (0.94)	4.41 (1.78)
S. sanguis	6.24 (0.90)	5.94 (0.56)	6.24 (0.63)
S. oralis	6.28 (0.93)	6.23 (0.28)	6.10 (0.22)
-ve cocci	6.11 (1.09)	5.72 (0.28)	6.43 (0.66)
Veillonella	6.05 (1.08)	5.72 (0.28)	6.40 (0.65)
+ve bacilli	6.63 (0.62)	6.34 (0.39)	7.38 (0.37)
Actinomyces	6.60 (0.65)	6.31 (0.39)	7.32 (0.31)
Lactobacillus	3.23 (2.30)	0.90 (0.52)	4.69 (2.29)
-ve bacilli	5.99 (0.76)	5.92 (0.21)	6.04 (0.52)
Bacteroides	5.46 (0.84)	5.15 (0.42)	5.98 (0.57)
Total	7.23 (0.59)	6.83 (0.15)	7.51 (0.37)

Table 4.19. cont.

SUBJECT D

	NP, n=7 Mean (SD)	SP, n=8 Mean (SD)	SPM, n=8 Mean (SD)
+ve cocci	6.35 (0.59)	6.66 (1.14)	6.07 (0.35)
S. mutans	2.36 (1.12)	2.96 (0.50)	3.34 (1.68)
S. sanguis	5.89 (0.59)	6.31 (1.13)	5.19 (0.61)
S. oralis	6.08 (0.54)	6.34 (1.17)	5.80 (0.48)
-ve cocci	5.66 (1.10)	5.52 (1.37)	5.60 (0.38)
Veillonella	5.66 (1.10)	5.52 (1.37)	5.54 (0.39)
+ve bacilli	7.05 (1.07)	6.69 (1.18)	6.34 (0.38)
Actinomyces	7.05 (1.07)	6.69 (1.18)	6.34 (0.38)
Lactobacillus	3.08 (2.96)	1.58 (1.56)	3.16 (1.88)
-ve bacilli	5.77 (1.12)	5.75 (1.23)	5.64 (0.57)
Bacteroides	5.63 (0.92)	5.38 (1.20)	5.26 (0.34)
Total	7.22 (0.98)	7.08 (1.18)	6.66 (0.34)

SUBJECT E

	NP, n=8 Mean (SD)	SP, n=8 Mean (SD)	SPM, n=8 Mean (SD)
+ve cocci	6.34 (1.14)	6.73 (0.41)	6.65 (0.30)
S. mutans	5.34 (1.88)	5.18 (1.89)	6.62 (0.28)
S. sanguis	5.47 (0.73)	5.86 (1.12)	5.25 (0.53)
s. oralis	5.79 (0.96)	5.62 (0.94)	5.54 (0.28)
-ve cocci	5.82 (0.84)	5.74 (0.69)	5.52 (0.48)
Veillonella	5.68 (0.76)	5.63 (0.57)	5.52 (0.48)
+ve bacilli	6.91 (0.38)	6.96 (0.52)	6.39 (0.43)
Actinomyces	6.85 (0.36)	6.92 (0.52)	5.79 (0.38)
Lactobacillus	3.52 (2.30)	4.91 (1.36)	4.78 (1.84)
-ve bacilli	5.48 (0.92)	6.08 (1.35)	5.22 (0.57)
Bacteroides	5.45 (0.89)	5.70 (0.96)	5.19 (0.53)
Total	7.12 (0.54)	7.20 (0.51)	6.88 (0.31)

Table 4.19. cont.

SUBJECT F

	NP, n=8 Mean (SD)	SP, n=8 Mean (SD)
+ve cocci	7.57 (0.18)	7.40 (0.48)
S. mutans	1.02 (1.20)	1.26 (0.95)
S. sanguis	7.04 (0.31)	6.60 (0.98)
S. oralis	7.33 (0.26)	7.22 (0.47)
-ve cocci	7.04 (0.35)	6.84 (0.91)
Veillonella	6.87 (0.44)	6.80 (0.89)
+ve bacilli	7.91 (0.33)	7.81 (0.62)
Actinomyces	7.86 (0.30)	7.76 (0.60)
Lactobacillus	3.73 (3.76)	0.92 (1.29)
-ve bacilli	7.46 (0.34)	7.40 (0.42)
Bacteroides	7.29 (0.38)	6.94 (0.75)
Total	8.24 (0.16)	8.09 (0.53)

SUBJECT G

	NP, n=4 Mean (SD)	SP, n=4 Mean (SD)
+ve cocci	7.63 (0.41)	7.88 (0.05)
S. mutans	0.15 (0.10)	0.19 (0.06)
S. sanguis	7.27 (0.44)	6.57 (0.42)
S. oralis	7.12 (0.46)	7.45 (0.19)
-ve cocci	6.74 (0.56)	7.28 (0.23)
Veillonella	6.74 (0.56)	7.28 (0.23)
+ve bacilli	6.78 (0.34)	6.36 (0.33)
Actinomyces	6.78 (0.34)	6.36 (0.33)
Lactobacillus	1.43 (1.67)	1.55 (0.99)
-ve bacilli	6.71 (0.28)	6.24 (0.33)
Bacteroides	6.11 (0.58)	6.11 (0.16)
Total	7.78 (0.37)	7.96 (0.06)

Table 4.19. cont.

SUBJECTS A - E

	NP, n=37 Mean (SD)	SP, n=38 Mean (SD)	SPM, n=39 Mean (SD)
+ve cocci	6.53 (0.81)	6.52 (0.65)	6.65 (0.50)
S. mutans	2.57 (2.46)	2.56 (2.06)	4.84 (2.09)
S. sanguis	5.98 (0.72)	5.88 (0.87)	5.67 (0.78)
S. oralis	6.00 (0.96)	6.03 (0.81)	5.98 (0.59)
-ve cocci	5.94 (0.84)	5.59 (0.84)	5.94 (0.75)
Veillonella	5.90 (0.82)	5.55 (0.81)	5.92 (0.76)
+ve bacilli	6.86 (0.74)	6.58 (0.75)	6.78 (0.63)
Actinomyces	6.84 (0.74)	6.55 (0.76)	6.63 (0.72)
Lactobacillus	2.45 (2.49)	2.10 (2.07)	3.46 (2.31)
-ve bacilli	5.68 (0.81)	5.90 (0.94)	5.74 (0.67)
Bacteroides	5.48 (0.79)	5.52 (0.89)	5.62 (0.65)
Total	7.18 (0.68)	6.96 (0.69)	7.15 (0.53)

Table 4.20. Total microbial counts (log_{10} cfu/mm^2 enamel surface), and absolute counts of predominant cultivable plaque microflora isolated from enamel slabs, under each treatment condition, for Subjects A - E combined.

	Position	Side	Run	Subject	Treatment
+ve cocci	NS	NS	***	**	NS
S. mutans	NS	NS	*	**	***
S. sanguis	NS	*	***	**	**
S. oralis	NS	NS	***	***	**
-ve cocci	NS	NS	***	***	NS
Veillonella	NS	NS	***	***	NS
+ve bacilli	NS	NS	***	***	**
Actinomyces	NS	NS	***	***	***
Lactobacillus	NS	NS	*	***	NS
-ve bacilli	NS	NS	***	***	**
Bacteroides	NS	NS	***	***	NS
Total	NS	NS	***	***	NS

Table 4.21. Statistical analysis of effect of position, side, run, subject and treatment conditions on $\log_{10}$ microbial counts of predominant cultivable microflora, and total $\log_{10}$ microbial count, in plaque isolated from enamel slabs.

SECTION - NORMAL PLAQUE

Subject	ΔZ^a (SE)	SZ^b (SE)	LB^c (SE)
A	241.6	1.33	1.29
n = 8	(79.2)	(0.36)	(0.35)
B	175.1	1.63	1.54
n = 8	(86.9)	(0.44)	(0.49)
C	243.0	1.30	1.73
n = 8	(101.1)	(0.57)	(0.55)
D	216.0	2.35	2.31
n = 8	(111.3)	(0.86)	(0.86)
E	388.2	2.26	1.91
n = 8	(72.2)	(0.59)	(0.64)
F	336.5	3.13	3.02
n = 8	(127.3)	(0.34)	(0.40)
G	373.0	1.78	1.74
n = 4	(110.2)	(0.34)	(0.35)
Total	274.9	1.98	1.95
n = 52	(38.6)	(0.22)	(0.22)

^{a} = % vol min. x μm ; ^{b} = % vol min. ; ^{c} = % vol min.

Table 4.22. Demineralisation parameters obtained from enamel sections, under Protocol I (NP), for Subjects A - G.

SECTION - SUCROSE PLAQUE

Subject	ΔZ (SE)	SZ (SE)	LB (SE)
A n = 8	305.6 (90.5)	1.30 (0.25)	1.18 (0.26)
B n = 8	246.1 (99.6)	1.56 (0.44)	2.19 (0.81)
C n = 8	131.0 (55.8)	1.76 (0.54)	1.72 (0.53)
D n = 8	539.8 (202.5)	5.08 (2.45)	5.69 (2.40)
E n = 8	517.1 (174.5)	3.26 (1.61)	3.32 (1.61)
F n = 8	379.4 (174.7)	3.50 (0.84)	4.25 (0.84)
G n = 4	140.0 (79.4)	2.39 (0.84)	2.39 (0.84)
Total n = 52	336.8 (55.6)	2.72 (0.62)	3.01 (0.62)

Table 4.23. Demineralisation parameters obtained from enamel sections, under Protocol II (SP), for Subjects A - G.

SECTION - SPM

Subject	ΔZ (SE)	SZ (SE)	LB (SE)
A n = 8	844.1 (406.4)	3.93 (1.58)	5.25 (2.16)
B n = 8	460.6 (88.3)	2.53 (0.38)	3.39 (0.60)
C n = 8	255.8 (152.6)	3.73 (2.43)	3.72 (2.43)
D n = 8	526.8 (20.2)	2.59 (0.93)	4.86 (1.60)
E n = 8	917.0 (168.9)	5.62 (1.25)	7.63 (1.93)
F	-	-	-
G	-	-	-
Total n = 40	600.8 (105.4)	3.68 (0.66)	4.97 (0.82)

Table 4.24. Demineralisation parameters obtained from enamel sections, under Protocol III (SPM), for Subjects A - E.

SECTION - ALL CONDITIONS

Subject	Δz (SE)	SZ (SE)	LB (SE)
A n = 24	463.8 (146.1)	2.19 (0.58)	2.57 (0.80)
B n = 24	294.0 (56.7)	1.91 (0.25)	2.37 (0.39)
C n = 24	209.9 (62.0)	2.27 (0.84)	2.39 (0.83)
D n = 24	427.5 (102.4)	3.34 (0.92)	4.29 (1.00)
E n = 24	607.5 (93.9)	3.71 (0.74)	4.29 (0.97)
F * n = 16	357.9 (104.5)	3.32 (1.29)	3.63 (1.25)
G * n = 8	256.5 (91.9)	2.08 (0.46)	2.06 (0.47)
Total n = 144	387.8 (39.3)	2.72 (0.30)	3.17 (0.34)

* - results refer to NP and SP groups only.

Table 4.25. Demineralisation parameters obtained from enamel sections, under all treatment conditions combined, for Subjects A - G.

	Position	Side	Run	Subject	Treatment
Δz	NS	NS	NS	NS	**
SZ	NS	NS	NS	NS	NS
LB	NS	*	NS	*	NS

NS = Not significant ; * = $p < 0.05$; ** = $p < 0.01$.

Table 4.26. Statistical analysis of effect of position, side, run, subject and treatment conditions on enamel demineralisation parameters.

Subject	Profile Group 1[a]	2[b]	3[c]	4[d]	Total
A	13	6	1	4	24
B	12	8	0	4	24
C	16	5	2	1	24
D	11	3	6	4	24
E	8	8	5	3	24
F*	10	2	1	3	16
G*	5	3	0	0	8

* - results refer to NP and SP groups only.

[a] = no change in profile from baseline ; [b] = slight surface softening ; [c] = more extensive surface softening ; [d] = subsurface demineralisation.

Table 4.27. Number of enamel sites in each profile group, and total number of enamel sites, for Subjects A - G.

Subject	Δz Group 1[a]	2[b]	3[c]	4[d]	Total
A	10	8	4	2	24
B	10	7	7	0	24
C	17	5	1	1	24
D	11	4	5	4	24
E	3	11	5	5	24
F*	7	5	3	1	16
G*	4	3	1	0	8

* = results refer to NP and SP groups only.

[a] = $\Delta z < 200$ units ; [b] = Δz between 200 and 499 units ;
[c] = Δz between 500 and 999 units ; [d] = $\Delta z > 1{,}000$ units.

Table 4.28. Number of enamel sites in each Δz group, and total number of enamel sites, for Subjects A - G.

	Profile Group			
Treatment	1	2	3	4
NP	33	14	2	3
SP	29	11	7	5
SPM	13	10	6	11
Total	75	35	15	19

Table 4.29. Number of enamel sites in each profile group, for each treatment condition.

	Δz Group			
Treatment	1	2	3	4
NP	25	17	10	0
SP	24	16	8	4
SPM	13	10	8	9
Total	62	43	26	13

Table 4.30. Number of enamel sites in each Δz group, for each treatment condition.

Table 4.31. Mean proportions of the predominant microflora associated with each Δz group, for each treatment condition.

NORMAL PLAQUE

	Δz Group 1 n = 25 Mean (SD)	2 n = 17 Mean (SD)	3 n = 10 Mean (SD)	4 n = 0 Mean (SD)
+ve cocci	34.4 (16.2)	40.0 (20.4)	38.6 (30.7)	nil
S. mutans	1.8 (8.3)	5.4 (12.1)	3.9 (11.8)	
S. sanguis	12.3 (11.5)	14.9 (15.4)	13.7 (15.7)	
S. oralis	15.2 (15.4)	14.9 (15.1)	17.1 (23.8)	
-ve cocci	11.6 (11.3)	11.0 (13.0)	4.0 (5.6)	nil
Veillonella	10.8 (11.2)	8.5 (12.4)	3.2 (5.5)	
+ve bacilli	47.5 (19.9)	40.5 (25.1)	52.1 (33.5)	nil
Actinomyces	41.1 (19.2)	32.2 (24.6)	46.3 (35.9)	
Lactobacillus	1.8 (3.7)	5.2 (13.1)	0.1 (0.1)	
-ve bacilli	6.7 (8.3)	8.5 (9.7)	5.4 (6.1)	nil
Bacteroides	3.7 (4.7)	3.6 (7.4)	0.8 (1.9)	

SUCROSE PLAQUE

	Δz Group 1 n = 24 Mean (SD)	2 n = 16 Mean (SD)	3 n = 8 Mean (SD)	4 n = 4 Mean (SD)
+ve cocci	43.0 (23.6)	46.2 (26.3)	33.4 (19.1)	40.6 (28.3)
S. mutans	2.8 (7.0)	0.6 (1.6)	4.6 (11.3)	3.3 (4.2)
S. sanguis	8.4 (10.6)	7.7 (10.8)	15.9 (20.6)	20.0 (27.6)
S. oralis	21.3 (24.1)	24.5 (22.4)	8.8 (11.8)	12.8 (6.6)
-ve cocci	8.1 (10.1)	6.4 (6.4)	8.6 (9.8)	12.9 (13.6)
Veillonella	8.0 (10.1)	6.2 (6.5)	7.6 (9.4)	11.7 (11.9)
+ve bacilli	41.3 (25.6)	35.8 (28.6)	51.0 (19.7)	43.8 (23.5)
Actinomyces	34.2 (25.2)	31.7 (24.1)	40.8 (12.3)	42.2 (22.4)
Lactobacillus	3.5 (9.9)	0.9 (3.6)	4.5 (6.6)	0.6 (1.0)
-ve bacilli	7.2 (8.6)	11.1 (10.9)	7.0 (7.9)	2.6 (3.4)
Bacteroides	3.0 (5.7)	5.5 (7.4)	5.2 (8.1)	ND

Table 4.31. cont.

SPM

	Δz Group 1 n = 13 Mean (SD)	2 n = 10 Mean (SD)	3 n = 8 Mean (SD)	4 n = 9 Mean (SD)
+ve cocci	47.0 (19.3)	45.0 (22.8)	37.6 (35.2)	39.1 (28.0)
S. mutans	8.9 (18.1)	22.8 (31.1)	27.7 (38.9)	30.5 (28.3)
S. sanguis	9.4 (11.6)	8.5 (11.8)	4.2 (8.7)	3.8 (8.5)
S. oralis	24.9 (17.1)	10.6 (11.6)	4.4 (6.4)	4.9 (9.7)
-ve cocci	12.2 (11.1)	9.9 (9.6)	8.6 (12.9)	3.2 (2.8)
Veillonella	11.8 (10.5)	9.6 (9.9)	8.6 (12.9)	3.2 (2.8)
+ve bacilli	36.9 (17.9)	40.9 (17.5)	51.0 (35.1)	54.8 (30.8)
Actinomyces	31.1 (16.2)	37.9 (17.8)	46.7 (34.0)	21.2 (26.7)
Lactobacillus	3.3 (4.6)	2.4 (3.4)	2.8 (3.6)	24.4 (21.6)
-ve bacilli	3.6 (4.8)	4.1 (5.4)	2.6 (2.3)	2.9 (4.4)
Bacteroides	2.1 (4.2)	3.5 (4.7)	1.8 (2.4)	0.5 (1.4)

Table 4.31. cont.

Table 4.32. Percentage isolation frequency of predominant organisms isolated from plaque associated with each Δz group, for each treatment condition.

NORMAL PLAQUE

	Δz Group 1 n = 25 F	2 n = 17 F	3 n = 10 F	4 n = 0 F
+ve cocci	100 %	100 %	100 %	nil
S. mutans	24 %	59 %	60 %	
S. sanguis	80 %	82 %	60 %	
S. oralis	76 %	79 %	60 %	
-ve cocci	84 %	65 %	50 %	nil
Veillonella	84 %	59 %	40 %	
+ve bacilli	100 %	100 %	100 %	nil
Actinomyces	100 %	94 %	90 %	
Lactobacillus	76 %	70 %	60 %	
-ve bacilli	64 %	70 %	60 %	nil
Bacteroides	56 %	35 %	20 %	

SUCROSE PLAQUE

	Δz Group 1 n = 24 F	2 n = 16 F	3 n = 8 F	4 n = 4 F
+ve cocci	100 %	100 %	100 %	100 %
S. mutans	62 %	56 %	75 %	100 %
S. sanguis	62 %	44 %	75 %	75 %
S. oralis	58 %	81 %	50 %	100 %
-ve cocci	67 %	75 %	75 %	75 %
Veillonella	67 %	75 %	75 %	75 %
+ve bacilli	100 %	100 %	100 %	100 %
Actinomyces	100 %	100 %	100 %	100 %
Lactobacillus	71 %	50 %	88 %	75 %
-ve bacilli	58 %	81 %	75 %	50 %
Bacteroides	29 %	44 %	62 %	ND

Table 4.32. cont.

SPM

	Δz Group			
	1	2	3	4
	n = 13	n = 10	n = 8	n = 9
	F	F	F	F
+ve cocci	100 %	100 %	100 %	100 %
S. mutans	85 %	80 %	88 %	100 %
S. sanguis	62 %	60 %	50 %	33 %
S. oralis	100 %	60 %	50 %	56 %
-ve cocci	85 %	80 %	75 %	78 %
Veillonella	85 %	70 %	75 %	78 %
+ve bacilli	100 %	100 %	100 %	100 %
Actinomyces	92 %	100 %	100 %	89 %
Lactobacillus	85 %	80 %	100 %	89 %
-ve bacilli	54 %	50 %	50 %	44 %
Bateroides	38 %	50 %	38 %	11 %

Table 4.32. cont.

Strep. mutans

NORMAL PLAQUE

	Δz Group			
	1	2	3	4
Median	ND	0.1	0.1	-
Mean	1.8	5.4	3.9	-
Range	ND - 41.7	ND - 40.0	ND - 37.5	-
n	25	17	10	0

SUCROSE PLAQUE

	Δz Group			
	1	2	3	4
Median	0.1	0.1	0.1	1.8
Mean	2.8	0.6	4.6	3.3
Range	ND - 26.3	ND - 5.6	ND - 32.3	0.1 - 9.5
n	24	16	8	4

SPM

	Δz Group			
	1	2	3	4
Median	0.1	0.1	0.1	33.3
Mean	8.9	22.8	27.7	30.5
Range	ND - 88.3	ND - 73.0	ND - 73.9	0.1 - 56.4
n	13	10	8	9

Table 4.33. Median, mean and range of proportions of Strep. mutans, in each Δz group, for each treatment condition.

Lactobacillus

NORMAL PLAQUE

	Δz Group			
	1	2	3	4
Median	0.1	0.1	0.1	-
Mean	1.8	5.2	0.1	-
Range	ND - 13.8	ND - 46.4	ND - 0.1	-
n	25	17	10	0

SUCROSE PLAQUE

	Δz Group			
	1	2	3	4
Median	0.1	0.1	1.6	0.1
Mean	3.5	0.9	4.5	0.6
Range	ND - 41.4	ND - 14.3	ND - 20.0	ND - 2.1
n	24	16	8	4

SPM

	Δz Group			
	1	2	3	4
Median	0.1	0.1	2.8	23.9
Mean	3.3	2.4	2.8	24.4
Range	ND - 7.4	ND - 8.6	0.1 - 12.5	ND - 49.0
n	13	10	8	9

Table 4.34. Median, mean and range of proportions of Lactobacillus, in each Δz group, for each treatment condition.

Table 4.35. % predominant cultivable plaque microflora isolated from enamel sites in each lesion profile group, for all Subjects and all treatment conditions combined.

PROFILE 1
ALL SUBJECTS

	F	Mean (SD)	Median	Range
+ve cocci	75/75	40.5 (21.3)	37.5	3.4 - 93.1
S. mutans	39/75	5.0 (15.7)	0.1	ND - 88.3
S. sanguis	53/75	10.4 (11.1)	6.9	ND - 46.2
S. oralis	57/75	18.4 (18.8)	14.3	ND - 69.6
-ve cocci	58/75	10.4 (10.6)	7.4	ND - 41.7
Veillonella	56/75	9.8 (10.6)	7.1	ND - 41.7
+ve bacilli	75/75	41.3 (23.3)	41.7	0.5 - 93.1
Actinomyces	74/75	35.2 (22.1)	31.0	ND - 93.0
Lactobacillus	53/75	3.1 (8.7)	0.1	ND - 46.4
-ve bacilli	48/75	7.6 (9.0)	4.9	ND - 40.0
Bacteroides	35/75	3.9 (5.8)	ND	ND - 28.6

PROFILE 2
ALL SUBJECTS

	F	Mean (SD)	Median	Range
+ve cocci	34/35	47.9 (24.4)	48.1	ND - 86.9
S. mutans	22/35	9.0 (19.4)	0.1	ND - 73.0
S. sanguis	21/35	12.3 (15.2)	6.2	ND - 60.6
S. oralis	23/35	18.8 (21.1)	11.8	ND - 76.9
-ve cocci	22/35	7.1 (9.6)	4.0	ND - 40.4
Veillonella	20/35	6.0 (8.7)	3.3	ND - 40.4
+ve bacilli	35/35	38.0 (25.8)	37.5	0.1 - 94.7
Actinomyces	34/35	32.2 (24.8)	29.6	ND - 94.7
Lactobacillus	24/35	1.6 (3.1)	0.1	ND - 12.5
-ve bacilli	23/35	6.7 (7.3)	4.2	ND - 29.4
Bacteroides	13/35	3.0 (5.3)	ND	ND - 20.8

PROFILE 3
ALL SUBJECTS

	F	Mean (SD)	Median	Range
+ve cocci	15/15	33.3 (23.2)	33.3	0.1 - 73.9
S. mutans	14/15	12.3 (23.6)	1.2	ND - 73.9
S. sanguis	6/15	7.6 (16.5)	ND	ND - 61.3
S. oralis	10/15	11.2 (13.5)	4.8	ND - 39.3
-ve cocci	12/15	8.8 (9.9)	5.1	ND - 29.4
Veillonella	12/15	8.5 (9.2)	5.1	ND - 29.4
+ve bacilli	15/15	54.6 (22.8)	47.0	23.9 -100.0
Actinomyces	14/15	45.7 (27.8)	43.3	ND -100.0
Lactobacillus	13/15	8.4 (14.6)	2.2	ND - 44.2
-ve bacilli	6/15	3.2 (6.4)	ND	ND - 24.3
Bacteroides	2/15	2.0 (6.3)	ND	ND - 24.3

PROFILE 4
ALL SUBJECTS

	F	Mean (SD)	Median	Range
+ve cocci	19/19	32.8 (25.0)	32.0	3.4 - 82.1
S. mutans	17/19	14.0 (22.8)	0.1	ND - 70.9
S. sanguis	12/19	9.6 (14.6)	3.9	ND - 60.7
S. oralis	11/19	7.0 (9.4)	3.4	ND - 34.3
-ve cocci	14/19	6.6 (9.6)	3.1	ND - 39.3
Veillonella	13/19	6.2 (9.6)	3.1	ND - 39.3
+ve bacilli	19/19	57.3 (26.2)	62.8	10.7 - 96.6
Actinomyces	19/19	40.3 (25.2)	38.3	2.0 - 79.3
Lactobacillus	17/19	8.6 (15.8)	0.1	ND - 49.0
-ve bacilli	11/19	3.5 (4.2)	1.8	ND - 13.0
Bacteroides	5/19	1.0 (1.9)	ND	ND - 6.2

Table 4.35. cont.

Table 4.36. % predominant cultivable plaque microflora isolated from enamel sites in each Δz group, for all treatment conditions combined, for Subjects A - G.

Δz GROUP 1[a]
SUBJECT A

	F	Mean (SD)	Median	Range
+ve cocci	10/10	51.9 (24.1)	49.0	14.3 - 91.3
S. mutans	4/10	21.1 (35.4)	ND	ND - 88.3
S. sanguis	6/10	11.2 (14.2)	4.0	ND - 36.5
S. oralis	7/10	13.5 (17.2)	5.4	ND - 46.7
-ve cocci	9/10	8.8 (13.1)	3.6	ND - 41.7
Veillonella	9/10	8.8 (13.1)	3.6	ND - 41.7
+ve bacilli	10/10	34.0 (25.3)	29.3	0.5 - 77.1
Actinomyces	10/10	29.6 (23.5)	25.0	0.5 - 77.1
Lactobacillus	7/10	0.07 (0.05)	0.1	ND - 0.1
-ve bacilli	8/10	4.8 (4.2)	4.6	ND - 11.1
Bacteroides	6/10	2.9 (4.0)	1.0	ND - 11.1

Δz GROUP 2[b]
SUBJECT A

	F	Mean (SD)	Median	Range
+ve cocci	8/8	59.9 (20.6)	67.2	26.7 - 86.9
S. mutans	6/8	13.4 (26.4)	0.1	ND - 70.9
S. sanguis	6/8	11.4 (10.6)	10.8	ND - 26.7
S. oralis	7/8	26.0 (24.1)	17.0	ND - 59.6
-ve cocci	6/8	8.9 (9.6)	5.2	ND - 26.7
Veillonella	6/8	8.9 (9.6)	5.2	ND - 26.7
+ve bacilli	8/8	20.9 (12.6)	22.2	3.0 - 40.0
Actinomyces	8/8	16.9 (12.2)	16.2	3.0 - 40.0
Lactobacillus	5/8	0.8 (2.1)	0.1	ND - 6.1
-ve bacilli	7/8	9.7 (13.0)	6.5	ND - 40.0
Bacteroides	3/8	4.5 (8.0)	ND	ND - 20.0

[a] - Δz < 200 units ; [b] - Δz between 200 and 499 units.

Δz GROUP 3[c]
SUBJECT A

	F	Mean (SD)	Median	Range
+ve cocci	4/4	42.1 (22.7)	40.2	16.7 - 71.4
S. mutans	3/4	5.0 (9.8)	0.1	ND - 19.6
S. sanguis	3/4	16.1 (20.5)	9.4	ND - 45.7
S. oralis	4/4	17.4 (12.4)	18.2	3.3 - 29.7
-ve cocci	3/4	3.7 (3.7)	3.0	ND - 8.9
Veillonella	3/4	3.7 (3.7)	3.0	ND - 8.9
+ve bacilli	4/4	47.4 (25.6)	41.9	25.7 - 80.0
Actinomyces	4/4	39.3 (23.1)	29.8	24.3 - 73.4
Lactobacillus	2/4	4.1 (5.9)	2.0	ND - 12.5
-ve bacilli	2/4	6.8 (11.8)	1.4	ND - 24.3
Bacteroides	1/4	6.1 (12.2)	ND	ND - 24.3

Δz GROUP 4[d]
SUBJECT A

	F	Mean (SD)	Median	Range
+ve cocci	2/2	63.2 (4.5)	63.2	60.0 - 66.4
S. mutans	2/2	52.4 (5.7)	52.4	48.4 - 56.4
S. sanguis	2/2	3.8 (0.2)	3.8	3.6 - 3.9
S. oralis	1/2	2.8 (3.9)	2.8	ND - 5.5
-ve cocci	2/2	2.4 (0.9)	2.4	1.8 - 3.1
Veillonella	2/2	2.4 (0.9)	2.4	1.8 - 3.1
+ve bacilli	2/2	33.6 (6.6)	33.6	28.9 - 38.2
Actinomyces	2/2	21.8 (15.4)	21.8	10.9 - 32.7
Lactobacillus	2/2	6.3 (8.8)	6.3	0.1 - 12.5
-ve bacilli	1/2	0.8 (1.1)	0.8	ND - 1.6
Bacteroides	0/2	ND	ND	ND

[c] - Δz between 500 and 1,000 units ; [d] - Δz > 1,000 units.

Table 4.36. cont.

Δz GROUP 1
SUBJECT B

	F	Mean (SD)	Median	Range
+ve cocci	10/10	33.0 (11.6)	33.9	7.3 - 45.6
S. mutans	3/10	1.5 (4.7)	ND	ND - 15.0
S. sanguis	8/10	7.4 (7.2)	6.2	ND - 24.1
S. oralis	10/10	22.6 (11.8)	23.0	2.4 - 37.1
-ve cocci	8/10	13.8 (12.4)	12.2	ND - 37.1
Veillonella	8/10	12.8 (12.0)	10.4	ND - 34.3
+ve bacilli	10/10	43.3 (18.9)	39.4	21.7 - 87.8
Actinomyces	10/10	42.7 (19.4)	39.4	21.7 - 87.8
Lactobacillus	5/10	0.05 (0.05)	0.05	ND - 0.1
-ve bacilli	9/10	8.9 (6.1)	9.6	ND - 19.6
Bacteroides	9/10	7.2 (5.2)	6.6	ND - 15.2

Δz GROUP 2
SUBJECT B

	F	Mean (SD)	Median	Range
+ve cocci	7/7	30.3 (13.7)	37.0	10.5 - 47.0
S. mutans	1/7	0.01 (0.04)	ND	ND - 0.1
S. sanguis	5/7	9.4 (13.0)	4.0	ND - 35.3
S. oralis	7/7	19.2 (8.1)	18.5	10.5 - 30.8
-ve cocci	5/7	7.8 (5.6)	11.1	ND - 12.5
Veillonella	5/7	6.7 (5.4)	8.0	ND - 12.5
+ve bacilli	7/7	49.9 (14.3)	41.2	36.0 - 70.8
Actinomyces	7/7	43.7 (10.0)	40.7	34.6 - 62.5
Lactobacillus	1/7	1.2 (3.1)	ND	ND - 8.3
-ve bacilli	6/7	11.4 (5.7)	11.8	ND - 18.5
Bacteroides	5/7	9.2 (7.1)	11.1	ND - 18.5

Table 4.36. cont.

Δz GROUP 3
SUBJECT B

	F	Mean (SD)	Median	Range
+ve cocci	6/7	20.0 (14.9)	27.6	ND - 35.7
S. mutans	4/7	0.06 (0.06)	0.1	ND - 0.1
S. sanguis	5/7	9.9 (9.0)	10.3	ND - 25.0
S. oralis	4/7	9.3 (9.1)	10.7	ND - 20.0
-ve cocci	6/7	12.9 (12.5)	10.3	ND - 39.3
Veillonella	6/7	11.8 (12.6)	8.6	ND - 39.3
+ve bacilli	7/7	62.2 (25.3)	62.8	21.4 - 94.7
Actinomyces	7/7	59.5 (25.0)	54.2	21.4 - 94.7
Lactobacillus	5/7	0.5 (1.3)	0.1	ND - 3.4
-ve bacilli	6/7	4.8 (3.4)	3.6	ND - 10.3
Bacteroides	4/7	2.8 (2.8)	3.4	ND - 6.9

Table 4.36. cont.

Δz GROUP 1
SUBJECT C

	F	Mean (SD)	Median	Range
+ve cocci	17/17	54.3 (14.4)	51.8	27.8 - 78.3
S. mutans	12/17	0.07 (0.05)	0.1	ND - 0.1
S. sanguis	13/17	14.8 (13.5)	14.8	ND - 46.2
S. oralis	17/17	36.4 (19.9)	31.5	3.8 - 69.6
-ve cocci	12/17	10.1 (11.7)	4.3	ND - 38.7
Veillonella	12/17	10.1 (11.7)	4.3	ND - 38.7
+ve bacilli	17/17	29.1 (16.5)	22.7	10.8 - 55.6
Actinomyces	17/17	27.6 (15.3)	22.7	10.8 - 55.6
Lactobacillus	14/17	0.3 (1.0)	0.1	ND - 4.2
-ve bacilli	7/17	6.8 (9.6)	ND	ND - 29.2
Bacteroides	1/17	1.2 (5.0)	ND	ND - 20.8

Δz GROUP 2
SUBJECT C

	F	Mean (SD)	Median	Range
+ve cocci	5/5	58.1 (11.7)	60.9	39.3 - 69.7
S. mutans	5/5	0.4 (0.6)	0.1	0.1 - 1.5
S. sanguis	4/5	26.3 (22.1)	24.0	ND - 60.6
S. oralis	5/5	27.5 (12.2)	32.0	9.1 - 39.3
-ve cocci	5/5	11.5 (4.6)	11.8	6.1 - 17.4
Veillonella	5/5	11.5 (4.6)	11.8	6.1 - 17.4
+ve bacilli	5/5	22.1 (14.4)	21.7	6.1 - 42.8
Actinomyces	5/5	20.3 (14.9)	13.0	6.1 - 42.8
Lactobacillus	4/5	1.8 (3.8)	0.1	ND - 8.6
-ve bacilli	4/5	8.3 (7.1)	8.0	ND - 18.2
Bacteroides	0/5	ND	ND	ND

Table 4.36. cont.

Δz GROUP 3
SUBJECT C

	F	% Count
+ve cocci	1/1	82.4
S. mutans	1/1	0.1
S. sanguis	1/1	23.5
S. oralis	1/1	23.5
-ve cocci	0/1	ND
Veillonella	0/1	ND
+ve bacilli	1/1	5.9
Actinomyces	1/1	5.9
Lactobacillus	1/1	0.1
-ve bacilli	1/1	11.8
Bacteroides	1/1	5.9

Δz GROUP 4
SUBJECT C

	F	% Count
+ve cocci	1/1	56.5
S. mutans	1/1	1.3
S. sanguis	1/1	26.1
S. oralis	1/1	30.4
-ve cocci	1/1	4.3
Veillonella	1/1	4.3
+ve bacilli	1/1	30.4
Actinomyces	1/1	30.4
Lactobacillus	0/1	ND
-ve bacilli	1/1	8.7
Bacteroides	0/1	ND

Table 4.36. cont.

Δz GROUP 1
SUBJECT D

	F	Mean (SD)	Median	Range
+ve cocci	11/11	27.2 (15.0)	25.0	6.9 - 57.7
S. mutans	7/11	0.3 (0.8)	0.1	ND - 2.7
S. sanguis	8/11	7.0 (5.3)	7.4	ND - 15.4
S. oralis	9/11	14.7 (14.2)	13.8	ND - 50.0
-ve cocci	9/11	8.4 (8.2)	7.4	ND - 28.9
Veillonella	9/11	7.6 (7.1)	7.4	ND - 23.7
+ve bacilli	11/11	61.3 (18.4)	61.5	34.6 - 93.1
Actinomyces	11/11	55.8 (21.8)	53.6	30.7 - 93.0
Lactobacillus	9/11	2.1 (2.4)	2.6	ND - 7.4
-ve bacilli	4/11	3.1 (5.1)	ND	ND - 14.3
Bacteroides	4/11	2.2 (3.7)	ND	ND - 11.1

Δz GROUP 2
SUBJECT D

	F	Mean (SD)	Median	Range
+ve cocci	4/4	33.1 (23.6)	34.5	2.9 - 60.5
S. mutans	3/4	0.08 (0.05)	0.1	ND - 0.1
S. sanguis	2/4	7.4 (9.3)	5.2	ND - 19.2
S. oralis	3/4	23.0 (23.2)	21.0	ND - 50.0
-ve cocci	3/4	13.9 (14.5)	13.1	ND - 29.4
Veillonella	2/4	13.1 (15.4)	11.6	ND - 29.4
+ve bacilli	4/4	47.9 (14.6)	44.8	34.6 - 67.6
Actinomyces	4/4	46.2 (14.6)	44.7	30.7 - 64.7
Lactobacillus	3/4	0.8 (1.4)	0.1	ND - 2.9
-ve bacilli	2/4	5.0 (6.2)	3.8	ND - 12.5
Bacteroides	2/4	3.5 (4.1)	3.1	ND - 7.7

Table 4.36. cont.

Δz GROUP 3
SUBJECT D

	F	Mean (SD)	Median	Range
+ve cocci	5/5	28.9 (27.1)	24.0	1.2 - 64.5
S. mutans	4/5	0.3 (0.5)	0.1	ND - 1.2
S. sanguis	3/5	18.7 (26.9)	2.6	ND - 61.3
S. oralis	3/5	6.5 (10.0)	3.2	ND - 24.0
-ve cocci	1/5	1.0 (2.3)	ND	ND - 5.1
Veillonella	1/5	1.0 (2.3)	ND	ND - 5.1
+ve bacilli	5/5	69.1 (25.5)	76.0	35.5 -100.0
Actinomyces	5/5	61.9 (30.3)	71.8	29.6 -100.0
Lactobacillus	4/5	2.9 (3.2)	3.2	ND - 7.7
-ve bacilli	1/5	1.0 (2.3)	ND	ND - 5.1
Bacteroides	1/5	1.0 (2.3)	ND	ND - 5.1

Δz GROUP 4
SUBJECT D

	F	Mean (SD)	Median	Range
+ve cocci	4/4	30.8 (37.0)	18.8	3.4 - 82.1
S. mutans	4/4	0.4 (0.5)	0.1	0.1 - 1.1
S. sanguis	2/4	18.5 (28.8)	6.6	ND - 60.7
S. oralis	4/4	8.8 (6.6)	7.1	3.4 - 17.8
-ve cocci	2/4	5.5 (9.7)	1.0	ND - 20.0
Veillonella	2/4	5.5 (9.7)	1.0	ND - 20.0
+ve bacilli	4/4	60.3 (39.5)	67.0	10.7 - 96.6
Actinomyces	4/4	42.9 (28.2)	40.8	10.7 - 79.3
Lactobacillus	3/4	15.7 (23.1)	6.9	ND - 48.9
-ve bacilli	2/4	3.4 (3.9)	3.2	ND - 7.1
Bacteroides	1/4	1.0 (2.1)	ND	ND - 4.2

Table 4.36. cont.

Δz GROUP 1
SUBJECT E

	F	Mean (SD)	Median	Range
+ve cocci	3/3	33.2 (18.6)	26.3	19.0 - 54.2
S. mutans	3/3	29.0 (11.6)	26.3	19.0 - 41.7
S. sanguis	0/3	ND	ND	ND
S. oralis	0/3	ND	ND	ND
-ve cocci	2/3	11.6 (10.2)	15.8	ND - 19.0
Veillonella	2/3	11.6 (10.2)	15.8	ND - 19.0
+ve bacilli	3/3	53.8 (10.7)	57.9	41.7 - 61.9
Actinomyces	3/3	46.1 (16.4)	47.3	29.2 - 61.9
Lactobacillus	3/3	4.2 (4.1)	4.1	0.1 - 8.3
-ve bacilli	1/3	1.4 (2.4)	ND	ND - 4.2
Bacteroides	1/3	1.4 (2.4)	ND	ND - 4.2

Δz GROUP 2
SUBJECT E

	F	Mean (SD)	Median	Range
+ve cocci	11/11	35.1 (22.1)	37.0	0.1 - 73.3
S. mutans	11/11	19.6 (25.0)	3.6	0.1 - 73.0
S. sanguis	4/11	3.8 (7.0)	ND	ND - 20.0
S. oralis	4/11	5.6 (9.8)	ND	ND - 28.0
-ve cocci	6/11	5.6 (9.6)	3.3	ND - 32.0
Veillonella	3/11	2.1 (4.8)	ND	ND - 16.0
+ve bacilli	11/11	53.2 (25.4)	52.0	20.0 - 92.8
Actinomyces	10/11	46.6 (27.1)	44.0	ND - 87.5
Lactobacillus	10/11	4.7 (10.0)	0.1	ND - 32.1
-ve bacilli	6/11	6.1 (8.8)	3.6	ND - 28.6
Bacteroides	5/11	5.4 (8.7)	ND	ND - 28.6

Table 4.36. cont.

Δz GROUP 3
SUBJECT E

	F	Mean (SD)	Median	Range
+ve cocci	5/5	44.3 (26.0)	43.8	8.0 - 73.9
S. mutans	5/5	41.4 (26.8)	37.5	4.0 - 73.9
S. sanguis	2/5	2.0 (2.9)	ND	ND - 6.2
S. oralis	0/5	ND	ND	ND
-ve cocci	3/5	7.8 (12.3)	2.2	ND - 29.0
Veillonella	3/5	7.8 (12.3)	2.2	ND - 29.0
+ve bacilli	5/5	46.4 (22.1)	36.4	23.9 - 80.0
Actinomyces	5/5	37.6 (15.0)	35.4	21.7 - 60.0
Lactobacillus	5/5	7.2 (7.9)	4.7	0.1 - 20.0
-ve bacilli	2/5	1.4 (2.0)	ND	ND - 4.0
Bacteroides	1/5	0.6 (1.4)	ND	ND - 3.2

Δz GROUP 4
SUBJECT E

	F	Mean (SD)	Median	Range
+ve cocci	5/5	32.6 (17.4)	33.3	12.2 - 54.3
S. mutans	5/5	30.1 (19.1)	33.3	9.5 - 54.3
S. sanguis	0/5	ND	ND	ND
S. oralis	2/5	1.5 (2.2)	ND	ND - 4.8
-ve cocci	4/5	9.4 (11.2)	5.7	ND - 28.6
Veillonella	4/5	8.5 (9.1)	5.7	ND - 23.8
+ve bacilli	5/5	58.0 (17.4)	52.4	40.0 - 83.7
Actinomyces	4/5	11.9 (22.6)	2.4	ND - 52.4
Lactobacillus	5/5	31.8 (19.1)	40.0	2.1 - 49.0
-ve bacilli	0/5	ND	ND	ND
Bacteroides	0/5	ND	ND	ND

Table 4.36. cont.

Δz GROUP 1
SUBJECT F

	F	Mean (SD)	Median	Range
+ve cocci	7/7	21.5 (23.5)	16.7	3.4 - 70.0
S. mutans	3/7	0.7 (1.8)	ND	ND - 4.9
S. sanguis	4/7	6.1 (9.0)	2.7	ND - 24.4
S. oralis	2/7	8.3 (20.6)	ND	ND - 55.0
-ve cocci	5/7	6.8 (6.0)	5.0	ND - 13.9
Veillonella	5/7	5.5 (4.5)	5.0	ND - 10.8
+ve bacilli	7/7	56.6 (24.4)	63.4	5.0 - 75.9
Actinomyces	7/7	32.0 (19.5)	27.0	5.0 - 63.9
Lactobacillus	5/7	10.5 (14.7)	7.3	ND - 41.4
-ve bacilli	7/7	15.1 (9.1)	17.2	4.9 - 29.2
Bacteroides	4/7	5.3 (6.5)	2.8	ND - 15.0

Δz GROUP 2
SUBJECT F

	F	Mean (SD)	Median	Range
+ve cocci	5/5	35.7 (27.3)	25.0	7.1 - 77.3
S. mutans	1/5	1.1 (2.5)	ND	ND - 5.6
S. sanguis	3/5	6.7 (6.6)	7.1	ND - 13.9
S. oralis	4/5	20.5 (25.8)	12.5	ND - 63.6
-ve cocci	3/5	2.6 (2.6)	2.8	ND - 5.9
Veillonella	3/5	2.6 (2.6)	2.8	ND - 5.9
+ve bacilli	5/5	47.0 (34.9)	43.8	9.1 - 89.3
Actinomyces	5/5	34.1 (26.2)	25.0	9.1 - 75.0
Lactobacillus	2/5	9.3 (20.7)	ND	ND - 46.4
-ve bacilli	4/5	14.7 (14.7)	9.1	ND - 31.2
Bacteroides	2/5	1.6 (2.2)	ND	ND - 4.5

Table 4.36. cont.

Δz GROUP 3
SUBJECT F

	F	Mean (SD)	Median	Range
+ve cocci	3/3	30.3 (40.5)	9.7	4.3 - 76.9
S. mutans	2/3	0.1 (0.1)	0.1	ND - 0.1
S. sanguis	1/3	3.2 (5.6)	ND	ND - 9.7
S. oralis	1/3	25.6 (44.4)	ND	ND - 76.9
-ve cocci	3/3	9.4 (7.2)	7.7	3.2 - 17.4
Veillonella	2/3	6.9 (9.3)	3.2	ND - 17.4
+ve bacilli	3/3	48.6 (42.8)	65.2	0.1 - 80.6
Actinomyces	2/3	38.9 (34.4)	51.6	ND - 65.2
Lactobacillus	3/3	0.1 (0.0)	0.1	0.1
-ve bacilli	3/3	11.6 (4.7)	13.0	6.4 - 15.4
Bacteroides	1/3	1.1 (1.8)	ND	ND - 3.2

Δz GROUP 4
SUBJECT F

	F	% Count
+ve cocci	1/1	28.1
S. mutans	1/1	2.4
S. sanguis	1/1	6.2
S. oralis	1/1	18.8
-ve cocci	1/1	3.1
Veillonella	1/1	3.1
+ve bacilli	1/1	65.6
Actinomyces	1/1	62.5
Lactobacillus	1/1	0.1
-ve bacilli	1/1	3.1
Bacteroides	0/1	ND

Table 4.36. cont.

Δz GROUP 1
SUBJECT G

	F	Mean (SD)	Median	Range
+ve cocci	4/4	63.6 (19.2)	62.3	42.8 - 87.1
S. mutans	0/4	ND	ND	ND
S. sanguis	4/4	17.8 (11.8)	17.2	4.6 - 32.2
S. oralis	1/4	0.9 (1.8)	ND	ND - 3.6
-ve cocci	3/4	16.0 (13.5)	17.8	ND - 28.6
Veillonella	3/4	16.0 (13.5)	17.8	ND - 28.6
+ve bacilli	4/4	19.4 (11.7)	22.2	3.2 - 30.2
Actinomyces	4/4	10.8 (10.6)	8.0	2.3 - 25.0
Lactobacillus	4/4	7.8 (13.5)	1.6	0.1 - 27.9
-ve bacilli	1/4	0.9 (1.8)	ND	ND - 3.6
Bacteroides	1/4	0.9 (1.8)	ND	ND - 3.6

Δz GROUP 2
SUBJECT G

	F	Mean (SD)	Median	Range
+ve cocci	3/3	63.2 (20.2)	57.1	46.8 - 85.7
S. mutans	0/3	ND	ND	ND
S. sanguis	3/3	22.5 (12.2)	27.6	8.6 - 31.4
S. oralis	1/3	5.7 (9.9)	ND	ND - 17.1
-ve cocci	3/3	24.9 (17.6)	28.6	5.7 - 40.4
Veillonella	3/3	24.9 (17.6)	28.6	5.7 - 40.4
+ve bacilli	3/3	10.5 (3.3)	8.6	8.5 - 14.3
Actinomyces	3/3	8.5 (5.9)	8.6	2.5 - 14.3
Lactobacillus	3/3	0.8 (1.2)	0.1	0.1 - 2.1
-ve bacilli	1/3	1.4 (2.4)	ND	ND - 4.2
Bacteroides	1/3	0.7 (1.2)	ND	ND - 2.1

Table 4.36. cont.

Δz GROUP 3
SUBJECT G

	F	% Count
+ve cocci	1/1	50.0
S. mutans	0/1	ND
S. sanguis	1/1	32.5
S. oralis	1/1	17.5
-ve cocci	1/1	5.0
Veillonella	1/1	5.0
+ve bacilli	1/1	37.5
Actinomyces	1/1	2.5
Lactobacillus	1/1	0.1
-ve bacilli	1/1	7.5
Bacteroides	1/1	2.5

Table 4.36. cont.

Table 4.37. % total cultivable plaque microflora isolated from enamel sites in each Δz group, for all Subjects combined.

Δz GROUP 1
ALL SUBJECTS

	F	Mean (SD)	Median	Range
+ve cocci	62/62	41.5 (21.8)	41.4	3.4 - 91.3
S. mutans	32/62	5.2 (16.7)	0.1	ND - 88.3
S. sanguis	43/62	10.1 (11.2)	6.9	ND - 46.2
S. oralis	46/62	19.4 (19.7)	14.6	ND - 69.6
S. salivarius	13/62	4.2 (12.6)	ND	ND - 74.2
S. milleri	3/62	0.2 (1.2)	ND	ND - 7.4
Other Strep.	7/62	0.7 (2.4)	ND	ND - 12.5
ANO_2 Strep.	17/62	1.5 (3.2)	ND	ND - 15.4
Micrococcus/Staph.	2/62	0.1 (0.8)	ND	ND - 4.5
-ve cocci	48/62	10.3 (10.9)	7.2	ND - 41.7
Veillonella	48/62	9.8 (10.6)	6.4	ND - 41.7
Neisseria	7/62	0.4 (1.3)	ND	ND - 5.6
+ve bacilli	62/62	41.6 (23.1)	41.8	0.5 - 93.1
A. odontolyticus	50/62	17.7 (16.6)	14.6	ND - 79.3
A. v/n - cat. +ve	31/62	7.6 (11.5)	1.1	ND - 50.0
A. v/n - cat. -ve	33/62	9.1 (12.4)	3.0	ND - 45.7
Other Actinomyces	7/62	1.3 (7.7)	ND	ND - 60.0
Lactobacillus	47/62	2.4 (6.7)	0.1	ND - 41.4
Bifidobacterium	8/62	2.2 (6.8)	ND	ND - 37.8
Propionibacterium	3/62	0.2 (0.8)	ND	ND - 4.2
Arachnia	0/62	ND	ND	ND
Eubacterium	6/62	1.0 (4.3)	ND	ND - 31.6
Unidentified	3/62	0.3 (1.5)	ND	ND - 10.5
-ve bacilli	37/62	6.4 (7.8)	3.5	ND - 29.2
Bacteroides	26/62	3.1 (5.0)	ND	ND - 20.8
Fusobacterium	4/62	0.2 (0.9)	ND	ND - 5.0
Capnocytophaga	19/62	2.5 (5.9)	ND	ND - 29.2
Haemophilus	1/62	0.1 (0.5)	ND	ND - 3.6
Eikenella	2/62	0.1 (0.4)	ND	ND - 3.4
Unidentified	3/62	0.5 (2.2)	ND	ND - 13.7

Δz GROUP 2
ALL SUBJECTS

	F	Mean (SD)	Median	Range
+ve cocci	43/43	43.5 (22.9)	40.0	0.1 - 86.9
S. mutans	27/43	7.7 (18.4)	0.1	ND - 73.0
S. sanguis	27/43	10.7 (13.2)	4.2	ND - 68.6
S. oralis	31/43	17.5 (18.1)	11.8	ND - 63.6
S. salivarius	8/43	3.3 (12.2)	ND	ND - 77.1
S. milleri	3/43	0.2 (0.8)	ND	ND - 3.7
Other Strep.	7/43	2.1 (6.3)	ND	ND - 29.6
ANO_2 Strep.	8/43	1.8 (5.1)	ND	ND - 27.7
Micrococcus/Staph.	2/43	0.2 (0.7)	ND	ND - 4.3
-ve cocci	31/43	9.0 (10.2)	5.7	ND - 40.4
Veillonella	27/43	7.9 (9.8)	4.0	ND - 40.4
Neisseria	7/43	1.1 (3.2)	ND	ND - 16.0
+ve bacilli	43/43	38.8 (24.6)	38.5	3.0 - 92.8
A. odontolyticus	34/43	16.2 (15.9)	13.3	ND - 68.8
A. v/n - cat. +ve	19/43	6.9 (12.3)	ND	ND - 55.6
A. v/n - cat. -ve	27/43	7.0 (8.1)	5.9	ND - 26.9
Other Actinomyces	5/43	2.1 (7.0)	ND	ND - 37.0
Lactobacillus	28/43	3.0 (8.8)	0.1	ND - 46.4
Bifidobacterium	8/43	1.6 (5.2)	ND	ND - 28.6
Propionibacterium	0/43	ND	ND	ND
Arachnia	1/43	0.1 (1.0)	ND	ND - 6.2
Eubacterium	5/43	0.7 (1.6)	ND	ND - 6.2
Unidentified	2/43	0.2 (0.8)	ND	ND - 4.0
-ve bacilli	30/43	8.5 (9.6)	6.7	ND - 40.0
Bacteroides	18/43	4.3 (6.8)	ND	ND - 28.6
Fusobacterium	5/43	0.4 (1.3)	ND	ND - 5.9
Capnocytophaga	15/43	2.4 (5.4)	ND	ND - 25.0
Haemophilus	1/43	0.1 (0.5)	ND	ND - 3.1
Eikenella	2/43	0.2 (1.1)	ND	ND - 6.7
Unidentified	6/43	1.0 (3.0)	ND	ND - 13.3

Table 4.37. cont

Δz GROUP 3
ALL SUBJECTS

	F	Mean (SD)	Median	Range
+ve cocci	25/26	34.5 (25.9)	32.2	ND - 82.4
S. mutans	19/26	8.8 (19.8)	0.1	ND - 73.9
S. sanguis	16/26	11.6 (15.9)	5.6	ND - 61.3
S. oralis	14/26	10.9 (16.8)	3.2	ND - 76.9
S. salivarius	3/26	1.6 (6.9)	ND	ND - 35.3
S. milleri	2/26	0.8 (3.6)	ND	ND - 18.5
Other Strep.	0/26	ND	ND	ND
ANO_2 Strep.	2/26	0.8 (3.0)	ND	ND - 14.9
Micrococcus/Staph.	0/26	ND	ND	ND
-ve cocci	17/26	7.0 (9.5)	4.2	ND - 39.3
Veillonella	16/26	6.4 (9.4)	3.0	ND - 39.3
Neisseria	3/26	0.6 (1.8)	ND	ND - 4.0
+ve bacilli	26/26	53.5 (27.4)	53.6	0.1 -100.0
A. odontolyticus	22/26	20.0 (16.4)	16.9	ND - 64.5
A. v/n - cat. +ve	18/26	17.0 (21.4)	9.7	ND - 66.7
A. v/n - cat. -ve	16/26	8.4 (10.6)	5.1	ND - 36.0
Other Actinomyces	2/26	1.3 (4.8)	ND	ND - 4.8
Lactobacillus	21/26	2.8 (4.8)	0.1	ND - 20.0
Bifidobacterium	6/26	3.1 (8.1)	ND	ND - 29.0
Propionibacterium	0/26	ND	ND	ND
Arachnia	0/26	ND	ND	ND
Eubacterium	2/26	0.3 (1.1)	ND	ND - 5.0
Unidentified	3/26	0.8 (2.7)	ND	ND - 12.5
-ve bacilli	16/26	4.9 (6.0)	3.3	ND - 24.3
Bacteroides	10/26	2.5 (5.0)	ND	ND - 24.3
Fusobacterium	7/26	1.0 (1.6)	ND	ND - 4.3
Capnocytophaga	5/26	1.0 (2.6)	ND	ND - 11.5
Haemophilus	0/26	ND	ND	ND
Eikenella	1/26	0.1 (2.8)	ND	ND - 2.8
Unidentified	2/26	0.3 (1.1)	ND	ND - 4.3

Table 4.37. cont.

Δz GROUP 4
ALL SUBJECTS

	F	Mean (SD)	Median	Range
+ve cocci	13/13	38.2 (24.8)	33.3	3.4 - 82.1
S. mutans	13/13	20.0 (22.9)	9.5	0.1 - 56.4
S. sanguis	6/13	8.8 (17.4)	ND	ND - 60.7
S. oralis	9/13	7.6 (9.4)	4.2	ND - 30.4
S. salivarius	1/13	0.3 (1.0)	ND	ND - 3.6
S. milleri	0/13	ND	ND	ND
Other Strep.	0/13	ND	ND	ND
ANO_2 Strep.	3/13	1.7 (3.5)	ND	ND - 10.0
Micrococcus/Staph.	1/13	0.4 (1.3)	ND	ND - 4.8
-ve cocci	10/13	6.3 (8.6)	3.1	ND - 28.6
Veillonella	10/13	5.9 (7.6)	3.1	ND - 23.8
Neisseria	1/13	0.4 (1.3)	ND	ND - 4.8
+ve bacilli	13/13	53.4 (25.3)	47.0	10.7 - 96.6
A. odontolyticus	8/13	9.9 (14.6)	2.9	ND - 43.8
A. v/n - cat. +ve	6/13	5.7 (8.7)	ND	ND - 28.6
A. v/n - cat. -ve	7/13	11.4 (15.2)	2.0	ND - 48.3
Other Actinomyces	1/13	1.3 (4.8)	ND	ND - 17.2
Lactobacillus	11/13	18.0 (20.5)	12.5	ND - 49.0
Bifidobacterium	4/13	1.4 (2.4)	ND	ND - 7.1
Propionibacterium	0/13	ND	ND	ND
Arachnia	1/13	0.3 (0.9)	ND	ND - 3.4
Eubacterium	2/13	5.4 (13.3)	ND	ND - 38.1
Unidentified	1/13	0.2 (0.9)	ND	ND - 3.1
-ve bacilli	5/13	2.1 (3.2)	ND	ND - 8.7
Bacteroides	1/13	0.3 (1.2)	ND	ND - 4.2
Fusobacterium	0/13	ND	ND	ND
Capnocytophaga	2/13	0.8 (2.4)	ND	ND - 8.7
Haemophilus	1/13	0.2 (0.9)	ND	ND - 3.1
Eikenella	0/13	ND	ND	ND
Unidentified	2/13	0.7 (2.0)	ND	ND - 7.1

Table 4.37. cont.

	Lesion Profile	Δz Group
+ve cocci	NS	NS
S. mutans	NS	NS
S. sanguis	NS	NS
S. oralis	*	NS
-ve cocci	NS	NS
Veillonella	NS	NS
+ve bacilli	NS	NS
Actinomyces	NS	NS
Lactobacillus	NS	***
-ve bacilli	NS	NS
Bacteroides	NS	NS

NS = Not significant ; * = $p < 0.05$; *** = $p < 0.001$.

Table 4.38. Statistical analysis of relationship between predominant cultivable plaque microflora and enamel demineralisation.

	Run 1	Run 2	Run 3	Overall Mean
Subject A	4.3	4.4	4.4	4.4
Subject B	7.6	5.7	7.8	7.1
Subject C	6.0	6.4	6.7	6.4
Subject D	3.7	4.3	4.3	4.1
Subject E	6.4	7.6	6.9	6.9
Subject F	8.6	7.5	-	8.0
Subject G	7.4	-	-	7.4

Table 4.39. Mean number of exposures per day to fermentable carbohydrate, during each experimental run, and for all runs combined, for each Subject.

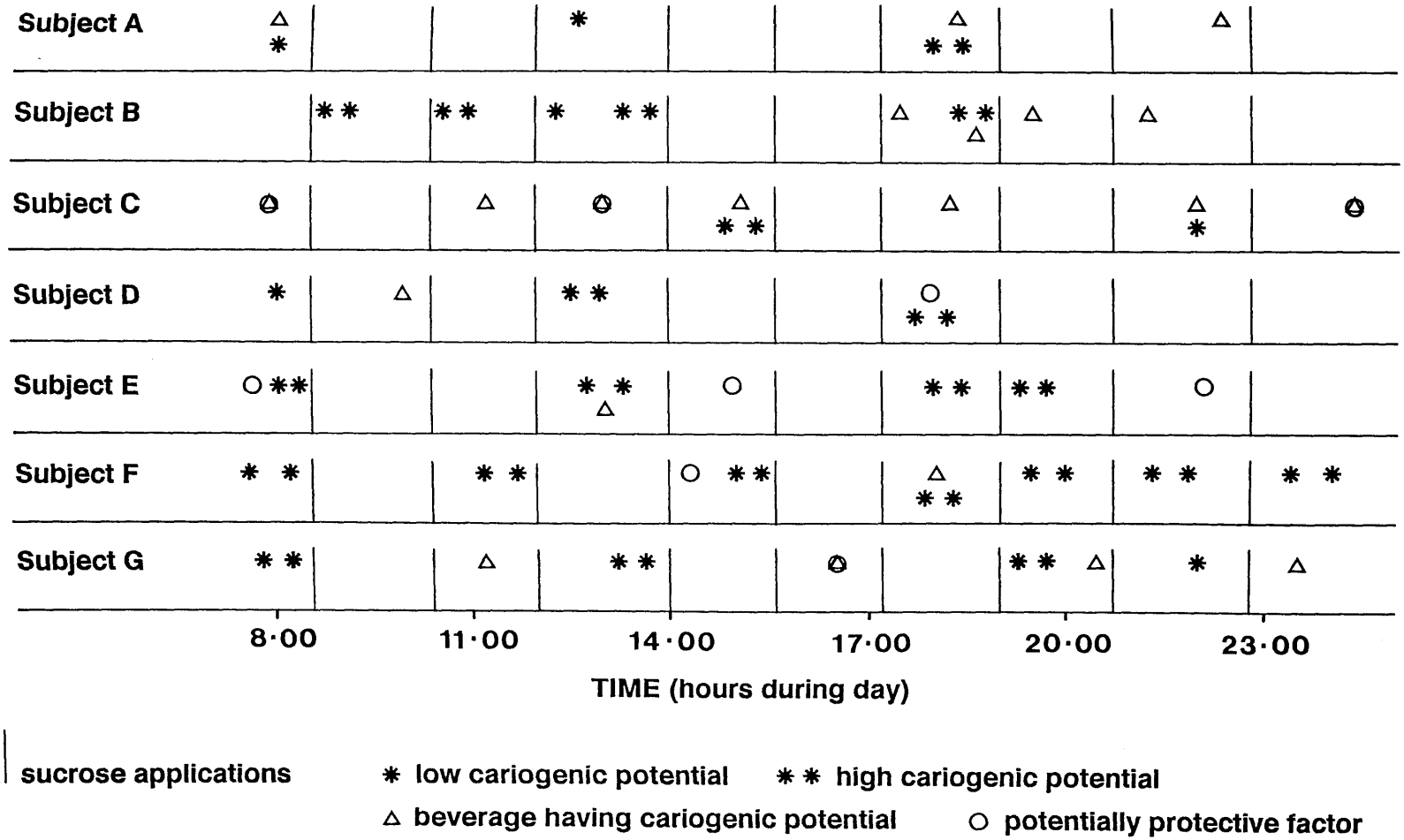


Fig. 4. Dietary assessment of "typical day" for each subject, showing intake of food and beverages, and applications of 10 % sucrose solution to appliance test sites.

NORMAL ENAMEL SECTION NP

	F	Mean (SD)	Median	Range
+ve cocci	9/9	55.3 (23.5)	45.2	19.2 - 93.1
S. mutans	3/9	2.3 (6.9)	ND	ND - 20.7
S. sanguis	6/9	20.8 (20.0)	5.6	ND - 48.3
S. oralis	9/9	27.4 (18.3)	35.1	2.9 - 53.5
-ve cocci	6/9	5.1 (5.5)	2.3	ND - 13.6
Veillonella	6/9	5.1 (5.5)	2.3	ND - 13.6
+ve bacilli	9/9	29.0 (18.4)	29.4	6.9 - 65.4
Actinomyces	9/9	27.0 (19.5)	23.5	6.9 - 65.4
Lactobacillus	2/9	0.4 (1.3)	ND	ND - 3.9
-ve bacilli	8/9	10.6 (9.1)	8.7	ND - 29.4
Bacteroides	8/9	9.2 (7.5)	8.7	ND - 21.4

ABRADED ENAMEL SECTION NP

	F	Mean (SD)	Median	Range
+ve cocci	9/9	55.9 (14.6)	53.6	37.0 - 75.9
S. mutans	4/9	1.0 (2.9)	ND	ND - 8.7
S. sanguis	9/9	30.8 (12.8)	34.6	14.3 - 50.0
S. oralis	8/9	17.1 (9.9)	19.2	ND - 27.3
-ve cocci	4/9	4.6 (7.6)	ND	ND - 22.5
Veillonella	4/9	3.8 (5.5)	ND	ND - 15.0
+ve bacilli	9/9	27.7 (10.8)	30.4	10.0 - 42.8
Actinomyces	9/9	24.2 (12.6)	23.0	6.5 - 42.8
Lactobacillus	1/9	0.01 (0.03)	ND	ND - 0.1
-ve bacilli	8/9	11.8 (10.0)	8.3	ND - 26.1
Bacteroides	7/9	8.4 (9.1)	3.8	ND - 25.0

Table 4.40. % predominant cultivable plaque microflora isolated from normal and abraded enamel section surfaces, under Protocol I (NP).

NORMAL ENAMEL SECTION SPM

	F	Mean (SD)	Median	Range
+ve cocci	9/9	56.2 (22.3)	60.9	8.2 - 82.5
S. mutans	9/9	23.3 (22.8)	18.2	0.1 - 68.4
S. sanguis	7/9	15.9 (18.4)	7.4	ND - 50.0
S. oralis	8/9	14.5 (11.3)	11.9	ND - 31.8
-ve cocci	5/9	5.8 (6.6)	6.1	ND - 19.0
Veillonella	5/9	5.4 (6.6)	3.0	ND - 19.0
+ve bacilli	8/9	30.8 (28.6)	27.3	ND - 91.8
Actinomyces	8/9	19.0 (13.1)	18.4	ND - 37.0
Lactobacillus	1/9	0.3 (0.8)	ND	ND - 2.5
-ve bacilli	7/9	7.2 (5.4)	9.5	ND - 15.2
Bacteroides	6/9	3.2 (3.3)	3.7	ND - 10.0

ABRADED ENAMEL SECTION SPM

	F	Mean (SD)	Median	Range
+ve cocci	9/9	64.3 (16.8)	68.8	28.2 - 82.1
S. mutans	9/9	39.4 (24.3)	44.9	0.3 - 68.8
S. sanguis	8/9	10.5 (9.9)	8.3	ND - 27.3
S. oralis	7/9	12.5 (14.5)	6.1	ND - 40.9
-ve cocci	8/9	8.0 (6.1)	5.0	ND - 18.8
Veillonella	7/9	7.6 (6.5)	5.0	ND - 18.8
+ve bacilli	9/9	22.5 (18.7)	28.8	3.6 - 66.7
Actinomyces	8/9	13.3 (10.2)	12.1	ND - 32.1
Lactobacillus	4/9	1.6 (3.0)	ND	ND - 9.1
-ve bacilli	8/9	5.2 (3.3)	5.0	ND - 10.7
Bacteroides	5/9	3.1 (3.4)	2.5	ND - 8.3

Table 4.41. % predominant cultivable plaque microflora isolated from normal and abraded enamel sections, under Protocol III (SPM).

NORMAL ENAMEL SLAB NP

	F	Mean (SD)	Median	Range
+ve cocci	6/6	46.4 (28.8)	52.0	2.4 - 78.8
S. mutans	2/6	0.03 (0.05)	ND	ND - 0.1
S. sanguis	4/6	20.8 (27.9)	6.6	ND - 63.6
S. oralis	4/6	18.6 (25.3)	14.0	ND - 68.2
-ve cocci	2/6	4.7 (10.0)	ND	ND - 25.0
Veillonella	2/6	4.7 (10.0)	ND	ND - 25.0
+ve bacilli	6/6	36.6 (22.4)	35.0	9.1 - 70.7
Actinomyces	6/6	28.6 (21.6)	22.5	9.1 - 70.7
Lactobacillus	2/6	3.7 (6.2)	ND	ND - 15.0
-ve bacilli	5/6	12.4 (9.9)	11.7	ND - 26.8
Bacteroides	5/6	10.7 (9.6)	8.4	ND - 26.8

ABRADED ENAMEL SLAB NP

	F	Mean (SD)	Median	Range
+ve cocci	6/6	24.5 (11.5)	24.3	9.4 - 40.0
S. mutans	2/6	0.03 (0.05)	ND	ND - 0.1
S. sanguis	5/6	12.5 (7.3)	13.4	ND - 20.0
S. oralis	2/6	6.7 (10.9)	ND	ND - 25.7
-ve cocci	3/6	2.4 (2.8)	1.6	ND - 6.2
Veillonella	3/6	2.4 (2.8)	1.6	ND - 6.2
+ve bacilli	6/6	61.7 (13.3)	64.3	37.1 - 75.0
Actinomyces	6/6	58.8 (14.5)	61.4	37.1 - 75.0
Lactobacillus	0/6	ND	ND	ND
-ve bacilli	5/6	11.5 (10.2)	8.1	ND - 25.0
Bacteroides	5/6	9.2 (8.4)	5.6	ND - 21.9

Table 4.42. % predominant cultivable plaque microflora isolated from normal and abraded enamel slab surfaces, under Protocol I (NP).

NORMAL ENAMEL SLAB SPM

	F	Mean (SD)	Median	Range
+ve cocci	6/6	45.6 (14.8)	44.8	22.2 - 66.7
S. mutans	6/6	11.7 (20.1)	4.4	0.1 - 51.8
S. sanguis	5/6	10.3 (13.3)	7.2	ND - 36.4
S. oralis	6/6	23.3 (12.9)	20.2	9.1 - 40.9
-ve cocci	5/6	8.1 (6.4)	7.4	ND - 18.2
Veillonella	5/6	6.8 (6.5)	5.8	ND - 18.2
+ve bacilli	6/6	38.6 (15.5)	33.0	26.5 - 66.7
Actinomyces	6/6	35.1 (19.7)	33.0	9.1 - 66.7
Lactobacillus	1/6	2.0 (4.9)	ND	ND - 12.1
-ve bacilli	5/6	7.6 (5.9)	7.4	ND - 17.6
Bacteroides	4/6	6.0 (6.6)	4.5	ND - 17.6

ABRADED ENAMEL SLAB SPM

	F	Mean (SD)	Median	Range
+ve cocci	6/6	48.4 (18.7)	51.6	25.0 - 76.5
S. mutans	6/6	7.7 (6.8)	7.8	0.1 - 16.7
S. sanguis	4/6	22.9 (24.7)	17.8	ND - 64.7
S. oralis	3/6	9.4 (15.7)	2.3	ND - 40.0
-ve cocci	4/6	6.5 (6.6)	5.1	ND - 15.0
Veillonella	3/6	4.2 (5.9)	2.2	ND - 15.0
+ve bacilli	6/6	32.3 (18.5)	30.7	7.8 - 62.5
Actinomyces	6/6	24.5 (15.0)	24.1	7.8 - 45.8
Lactobacillus	1/6	0.01 (0.04)	ND	ND - 0.1
-ve bacilli	6/6	12.8 (7.2)	11.2	5.0 - 26.1
Bacteroides	4/6	7.2 (8.5)	4.4	ND - 21.7

Table 4.43. % predominant cultivable plaque microflora isolated from normal and abraded enamel slab surfaces, under Protocol III (SPM).

	Surface	Treatment	Position	Side	Run
+ve cocci	NS	NS	NS	*	NS
S. mutans	NS	***	**	NS	NS
S. sanguis	NS	NS	NS	NS	NS
S. oralis	NS	NS	NS	NS	*
-ve cocci	NS	NS	*	NS	NS
Veillonella	NS	NS	*	NS	NS
+ve bacilli	NS	NS	NS	NS	*
Actinomyces	NS	**	NS	NS	***
Lactobacillus	NS	NS	NS	NS	NS
-ve bacilli	NS	*	NS	NS	**
Bacteroides	NS	***	NS	*	*

NS = Not significant ; * = $p < 0.05$; ** = $p < 0.01$; *** $p = < 0.001$.

Table 4.44. Statistical analysis of effect of enamel surface, treatment conditions, position, side and run on predominant cultivable plaque microflora.

NORMAL PLAQUE

	Normal Enamel Mean (SD)	Abraded Enamel Mean (SD)
+ve cocci	7.47 (0.75)	7.30 (0.56)
S. mutans	0.54 (0.50)	0.48 (0.47)
S. sanguis	6.92 (1.12)	6.96 (0.80)
S. oralis	6.91 (0.80)	6.71 (0.73)
-ve cocci	6.45 (0.60)	6.44 (0.64)
Veillonella	6.45 (0.60)	6.44 (0.64)
+ve bacilli	7.45 (0.39)	7.68 (0.60)
Actinomyces	7.35 (0.50)	7.66 (0.62)
Lactobacillus	2.36 (3.28)	0.21 (0.03)
-ve bacilli	6.98 (0.80)	6.89 (1.01)
Bacteroides	6.92 (0.76)	6.82 (0.96)
Total	7.95 (0.52)	7.90 (0.64)

Table 4.45. Counts (log_{10} cfu/mm^2 enamel surface) of predominant cultivable microflora, and total microbial counts, in plaque isolated from normal and abraded enamel slab surfaces, under Protocol I (NP), n=6.

SPM

	Normal Enamel Mean (SD)	Abraded Enamel Mean (SD)
+ve cocci	7.82 (0.52)	7.72 (0.54)
S. mutans	5.27 (2.68)	5.75 (2.24)
S. sanguis	7.05 (0.71)	7.11 (0.99)
S. oralis	7.50 (0.29)	6.74 (0.65)
-ve cocci	7.06 (0.60)	6.78 (0.53)
Veillonella	7.02 (0.58)	6.60 (0.68)
+ve bacilli	7.74 (0.38)	7.46 (0.44)
Actinomyces	7.66 (0.39)	7.35 (0.37)
Lactobacillus	1.49 (2.96)	0.57 (0.63)
-ve bacilli	7.01 (0.50)	7.12 (0.59)
Bacteroides	6.89 (0.55)	6.75 (0.79)
Total	8.17 (0.45)	8.00 (0.50)

Table 4.46. Counts (log_{10} cfu/mm^2 enamel surface) of predominant cultivable microflora, and total microbial counts, in plaque isolated from normal and abraded enamel slab surfaces, under Protocol III (SPM), n=6.

	Surface	Treatment	Position	Side	Run
+ve cocci	NS	NS	NS	NS	*
S. mutans	NS	***	NS	NS	NS
S. sanguis	NS	NS	NS	**	***
S. oralis	NS	NS	NS	NS	NS
-ve cocci	NS	*	NS	NS	*
Veillonella	NS	*	NS	NS	*
+ve bacilli	NS	NS	NS	NS	**
Actinomyces	NS	NS	NS	NS	**
Lactobacillus	NS	NS	NS	NS	NS
-ve bacilli	NS	NS	NS	NS	***
Bacteroides	NS	NS	NS	NS	***
Total	NS	NS	NS	NS	***

NS = Not significant ; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Table 4.47. Statistical analysis of effect of enamel surface, treatment conditions, position, side and run on $\log_{10}$ microbial counts of predominant cultivable plaque microflora.

NP
NORMAL ENAMEL

	Mean (SE)	Median	Range
Δz	98.9 (33.2)	119.0	0.0 - 246.0
SZ	0.27 (0.09)	0.17	0.0 - 0.67
LB	0.22 (0.09)	0.12	0.0 - 0.67

ABRADED ENAMEL

	Mean (SE)	Median	Range
Δz	210.2 (90.3)	144.0	0.0 - 819.0
SZ	1.17 (0.39)	0.77	0.0 - 2.74
LB	1.52 (0.18)	0.90	0.0 - 3.97

Table 4.48. Demineralisation parameters from normal and abraded enamel surfaces, under Protocol I (NP), n=9.

SPM
NORMAL ENAMEL

	Mean (SE)	Median	Range
Δz	143.9 (48.3)	147.0	0.0 - 399.0
SZ	2.06 (0.91)	1.06	0.0 - 6.98
LB	2.31 (1.04)	0.18	0.0 - 6.99

ABRADED ENAMEL

	Mean (SE)	Median	Range
Δz	1126.4(498.0)	638.0	0.0 -4598.0
SZ	7.73 (4.50)	0.19	0.0 - 33.34
LB	10.32 (4.67)	5.71	0.0 - 37.89

Table 4.49. Demineralisation parameters from normal and abraded enamel surfaces, under Protocol III (SPM), n=9.

BOTH CONDITIONS
NORMAL ENAMEL

	Mean (SE)	Median	Range
Δz	121.4 (41.1)	133.0	0.0 - 399.0
SZ	1.16 (0.70)	0.30	0.0 - 6.98
LB	1.27 (0.80)	0.14	0.0 - 6.99

ABRADED ENAMEL

	Mean (SE)	Median	Range
Δz	668.3(381.2)	262.0	0.0 -4598.0
SZ	4.45 (3.29)	0.64	0.0 - 33.34
LB	5.92 (3.56)	1.74	0.0 - 37.89

Table 4.50. Demineralisation parameters from normal and abraded enamel surfaces, under Protocols I and III combined, n=18.

	Surface	Treatment	Position	Side	Run
Δz	*	NS	NS	NS	NS
SZ	NS	NS	NS	NS	NS
LB	NS	NS	NS	NS	NS

NS = Not significant ; * = $p < 0.05$.

Table 4.51. Statistical analysis of effect of enamel surface, treatment conditions, position, side and run on enamel demineralisation parameters.

NORMAL PLAQUE

	Δz Group			
	1	2	3	4
Normal Enamel	8	1	0	0
Abraded Enamel	5	3	1	0

SPM

	Δz Group			
	1	2	3	4
Normal Enamel	6	3	0	0
Abraded Enamel	3	1	1	4

Table 4.52. Number of enamel sites in each Δz group, for normal and abraded sites, for all treatment conditions combined.

NORMAL PLAQUE

	Profile Group			
	1	2	3	4
Normal Enamel	8	1	0	0
Abraded Enamel	6	2	1	0

SPM

	Profile Group			
	1	2	3	4
Normal Enamel	6	3	0	0
Abraded Enamel	4	0	1	4

Table 4.53. Number of enamel sites in each lesion profile group, for normal and abraded sites, for all treatment conditions combined.

NORMAL ENAMEL NP + SPM
Δz GROUP 1

	F	Mean (SD)	Median	Range
+ve cocci	14/14	57.0 (20.3)	59.8	19.2 - 93.1
S. mutans	8/14	14.1 (21.8)	2.0	ND - 68.4
S. sanguis	10/14	20.9 (20.1)	19.5	ND - 50.0
S. oralis	13/14	18.4 (15.2)	15.6	ND - 43.5
-ve cocci	8/14	5.6 (6.4)	3.7	ND - 19.0
Veillonella	8/14	5.4 (6.4)	2.6	ND - 19.0
+ve bacilli	13/14	26.8 (19.1)	25.4	ND - 65.4
Actinomyces	13/14	23.2 (18.0)	21.3	ND - 65.4
Lactobacillus	3/14	0.5 (1.2)	ND	ND - 3.9
-ve bacilli	13/14	10.5 (7.4)	9.3	ND - 29.4
Bacteroides	12/14	7.0 (6.8)	4.9	ND - 21.4

NORMAL ENAMEL NP + SPM
Δz GROUP 2

	F	Mean (SD)	Median	Range
+ve cocci	4/4	51.2 (31.2)	57.7	8.2 - 81.4
S. mutans	4/4	8.3 (9.6)	7.4	0.1 - 18.2
S. sanguis	3/4	9.4 (11.2)	6.0	ND - 25.6
S. oralis	4/4	29.8 (18.7)	28.7	8.2 - 53.5
-ve cocci	3/4	4.7 (4.3)	4.8	ND - 9.1
Veillonella	3/4	4.7 (4.3)	4.8	ND - 9.1
+ve bacilli	4/4	40.8 (36.2)	32.6	7.0 - 91.8
Actinomyces	4/4	22.4 (12.8)	22.8	7.0 - 37.0
Lactobacillus	0/4	ND	ND	ND
-ve bacilli	2/4	3.2 (4.4)	1.8	ND - 9.3
Bacteroides	2/4	3.2 (4.4)	1.8	ND - 9.3

Table 4.54. % predominant cultivable microflora isolated from normal enamel sites, in each Δz group, for both treatment conditions combined.

Table 4.55. % predominant cultivable microflora isolated from abraded enamel sites, in each Δz group, for both treatment conditions combined.

ABRADED ENAMEL NP + SPM
Δz GROUP 1

	F	Mean (SD)	Median	Range
+ve cocci	8/8	59.9 (12.2)	61.8	38.1 - 72.7
S. mutans	4/8	20.7 (31.0)	0.1	ND - 68.8
S. sanguis	7/8	22.5 (19.3)	19.6	ND - 50.0
S. oralis	6/8	14.8 (11.9)	20.0	ND - 27.3
-ve cocci	5/8	6.1 (6.9)	4.0	ND - 18.8
Veillonella	5/8	6.1 (6.9)	4.0	ND - 18.8
+ve bacilli	8/8	26.1 (11.6)	22.8	9.4 - 42.8
Actinomyces	7/8	22.7 (14.7)	22.2	ND - 42.8
Lactobacillus	3/8	0.7 (1.3)	ND	ND - 3.1
-ve bacilli	7/8	7.8 (7.6)	6.6	ND - 25.0
Bacteroides	5/8	5.6 (8.4)	3.6	ND - 25.0

ABRADED ENAMEL NP + SPM
ΔZ GROUP 2

	F	Mean (SD)	Median	Range
+ve cocci	4/4	57.4 (20.4)	55.2	37.0 - 82.1
S. mutans	3/4	16.5 (27.4)	4.4	ND - 57.1
S. sanguis	4/4	23.6 (14.8)	24.9	7.1 - 37.5
S. oralis	4/4	13.9 (9.3)	11.8	5.0 - 26.9
-ve cocci	3/4	8.2 (9.9)	5.0	ND - 22.5
Veillonella	2/4	5.4 (7.1)	3.2	ND - 15.0
+ve bacilli	4/4	18.7 (14.0)	20.2	3.6 - 30.8
Actinomyces	4/4	12.7 (12.3)	8.2	3.6 - 30.8
Lactobacillus	1/4	0.02 (0.05)	ND	ND - 0.1
-ve bacilli	4/4	15.8 (10.3)	16.6	3.8 - 26.1
Bacteroides	4/4	11.4 (7.2)	11.0	3.8 - 19.6

ABRADED ENAMEL
NP + SPM
Δz GROUP 3

	F	Mean (SD)	Median	Range
+ve cocci	2/2	74.3 (2.3)	74.3	72.7 - 75.9
S. mutans	2/2	0.2 (0.1)	0.2	0.1 - 0.3
S. sanguis	2/2	24.0 (4.7)	24.0	20.7 - 27.3
S. oralis	2/2	27.4 (19.2)	27.4	13.8 - 40.9
-ve cocci	1/2	2.2 (3.2)	2.2	ND - 4.5
Veillonella	1/2	2.2 (3.2)	2.2	ND - 4.5
+ve bacilli	2/2	20.0 (3.9)	20.0	17.2 - 22.7
Actinomyces	2/2	20.0 (3.9)	20.0	17.2 - 22.7
Lactobacillus	0/2	ND	ND	ND
-ve bacilli	1/2	3.4 (4.9)	3.4	ND - 6.9
Bacteroides	1/2	1.7 (2.4)	1.7	ND - 3.4

ABRADED ENAMEL NP + SPM
Δz GROUP 4

	F	Mean (SD)	Median	Range
+ve cocci	4/4	56.1 (22.2)	58.4	28.2 - 79.6
S. mutans	4/4	33.0 (17.7)	31.4	17.8 - 51.5
S. sanguis	4/4	12.4 (9.9)	9.6	3.6 - 26.5
S. oralis	3/4	9.4 (13.0)	4.6	ND - 28.6
-ve cocci	3/4	6.9 (6.2)	6.6	ND - 14.3
Veillonella	3/4	6.9 (6.2)	6.6	ND - 14.3
+ve bacilli	4/4	32.1 (25.1)	26.6	8.2 - 66.7
Actinomyces	4/4	16.3 (10.7)	12.4	8.2 - 32.1
Lactobacillus	1/4	2.3 (4.6)	ND	ND - 9.1
-ve bacilli	4/4	5.0 (2.3)	4.4	3.0 - 8.2
Bacteroides	2/4	2.4 (3.0)	3.0	ND - 6.1

Table 4.55. cont.

Table 4.55. % predominant cultivable microflora isolated from abraded enamel sites, in each Δz group, for both treatment conditions combined.

ABRADED ENAMEL NP + SPM
Δz GROUP 1

	F	Mean (SD)	Median	Range
+ve cocci	8/8	59.9 (12.2)	61.8	38.1 - 72.7
S. mutans	4/8	20.7 (31.0)	0.1	ND - 68.8
S. sanguis	7/8	22.5 (19.3)	19.6	ND - 50.0
S. oralis	6/8	14.8 (11.9)	20.0	ND - 27.3
-ve cocci	5/8	6.1 (6.9)	4.0	ND - 18.8
Veillonella	5/8	6.1 (6.9)	4.0	ND - 18.8
+ve bacilli	8/8	26.1 (11.6)	22.8	9.4 - 42.8
Actinomyces	7/8	22.7 (14.7)	22.2	ND - 42.8
Lactobacillus	3/8	0.7 (1.3)	ND	ND - 3.1
-ve bacilli	7/8	7.8 (7.6)	6.6	ND - 25.0
Bacteroides	5/8	5.6 (8.4)	3.6	ND - 25.0

ABRADED ENAMEL NP + SPM
ΔZ GROUP 2

	F	Mean (SD)	Median	Range
+ve cocci	4/4	57.4 (20.4)	55.2	37.0 - 82.1
S. mutans	3/4	16.5 (27.4)	4.4	ND - 57.1
S. sanguis	4/4	23.6 (14.8)	24.9	7.1 - 37.5
S. oralis	4/4	13.9 (9.3)	11.8	5.0 - 26.9
-ve cocci	3/4	8.2 (9.9)	5.0	ND - 22.5
Veillonella	2/4	5.4 (7.1)	3.2	ND - 15.0
+ve bacilli	4/4	18.7 (14.0)	20.2	3.6 - 30.8
Actinomyces	4/4	12.7 (12.3)	8.2	3.6 - 30.8
Lactobacillus	1/4	0.02 (0.05)	ND	ND - 0.1
-ve bacilli	4/4	15.8 (10.3)	16.6	3.8 - 26.1
Bacteroides	4/4	11.4 (7.2)	11.0	3.8 - 19.6

ABRADED ENAMEL
NP + SPM
Δz GROUP 3

	F	Mean (SD)	Median	Range
+ve cocci	2/2	74.3 (2.3)	74.3	72.7 - 75.9
S. mutans	2/2	0.2 (0.1)	0.2	0.1 - 0.3
S. sanguis	2/2	24.0 (4.7)	24.0	20.7 - 27.3
S. oralis	2/2	27.4 (19.2)	27.4	13.8 - 40.9
-ve cocci	1/2	2.2 (3.2)	2.2	ND - 4.5
Veillonella	1/2	2.2 (3.2)	2.2	ND - 4.5
+ve bacilli	2/2	20.0 (3.9)	20.0	17.2 - 22.7
Actinomyces	2/2	20.0 (3.9)	20.0	17.2 - 22.7
Lactobacillus	0/2	ND	ND	ND
-ve bacilli	1/2	3.4 (4.9)	3.4	ND - 6.9
Bacteroides	1/2	1.7 (2.4)	1.7	ND - 3.4

ABRADED ENAMEL NP + SPM
Δz GROUP 4

	F	Mean (SD)	Median	Range
+ve cocci	4/4	56.1 (22.2)	58.4	28.2 - 79.6
S. mutans	4/4	33.0 (17.7)	31.4	17.8 - 51.5
S. sanguis	4/4	12.4 (9.9)	9.6	3.6 - 26.5
S. oralis	3/4	9.4 (13.0)	4.6	ND - 28.6
-ve cocci	3/4	6.9 (6.2)	6.6	ND - 14.3
Veillonella	3/4	6.9 (6.2)	6.6	ND - 14.3
+ve bacilli	4/4	32.1 (25.1)	26.6	8.2 - 66.7
Actinomyces	4/4	16.3 (10.7)	12.4	8.2 - 32.1
Lactobacillus	1/4	2.3 (4.6)	ND	ND - 9.1
-ve bacilli	4/4	5.0 (2.3)	4.4	3.0 - 8.2
Bacteroides	2/4	2.4 (3.0)	3.0	ND - 6.1

Table 4.55. cont.

	Lesion Profile	Δz Group
+ve cocci	NS	NS
S. mutans	NS	NS
S. sanguis	NS	NS
S. oralis	NS	NS
-ve cocci	NS	NS
Veillonella	NS	NS
+ve bacilli	NS	NS
Actinomyces	NS	NS
Lactobacillus	NS	NS
-ve bacilli	NS	NS
Bacteroides	NS	NS

NS = Not significant.

Table 4.56. Statistical analysis of relationship between predominant cultivable plaque microflora and enamel demineralisation, for abraded and non-abraded sites combined.

4.6. Discussion.

4.6.1. Subject Characteristics.

Primary carious enamel lesions occur predominantly during childhood, and this population group is therefore used when carrying out longitudinal and cross-sectional clinical studies investigating the microbial aetiology of the disease. Numerous such studies have been reported (Loesche et al., 1975; Hardie et al., 1977; Duchin & van Houte, 1978; Lang et al., 1987).

However, in experimental studies where the aim is to produce demineralisation as rapidly as possible in the oral environment, ethical problems arise regarding the use of child subjects, and their compliance and availability may be less than that obtained with adults. In this study, adult volunteers were therefore used, after being fully informed as to the requirements of the experimental periods. Nonetheless, the enamel sections used were obtained from children's premolars, as these teeth would have been exposed to the oral environment for fairly similar lengths of time, and may be more susceptible to demineralisation than older teeth.

Initial screening tests were carried out on the seven subjects in an attempt to assess their caries risk status, and hence the likelihood of demineralisation occurring on enamel sections in their mouths. In two subjects, the salivary flow rate was in the "low - normal range" and the buffering capacity "poor - intermediate", suggesting a higher caries susceptibility in these individuals (Krasse, 1985). One of these subjects had further risk factors, having relatively high Strep. mutans and Lactobacillus spp. salivary counts and the highest DMFS score of all the subjects. The other, however, had relatively low microbial counts and intermediate DMFS score.

When the various risk factors were taken together, six of the seven subjects were considered to be in the intermediate risk group, while the seventh was of high caries risk status.

However, assessment of caries risk status of an individual is often difficult as the previously mentioned factors, which may be conflicting,

together with diet and varying host resistance, are among the components that have to be taken into account regarding this multifactorial disease (Krasse, 1985).

4.6.2. Dietary Assessment of Subjects.

Dietary analysis of individuals is difficult and, while a diet history is the only practicable method of collecting data over a 7-day period, it has many limitations and should be regarded as an inexact assessment and thus interpreted with caution (Kidd and Joyston-Bechal, 1987).

The recording of dietary intake is dependent on the co-operation and honesty of volunteers and may be influenced by the fact that the individuals may change their normal eating pattern during a recording period, as they know their dietary habits will subsequently be analysed. In this study, subjects were each given a pocket-sized note-pad to encourage them to carry it around and to make recordings. The recording period spanned the middle week of the experimental period, and although only a third of the time was used to encourage compliance, it should have provided a fair representation of the appliance-wearing period as a whole.

Furthermore, the analysis of diet diaries is also extremely difficult as many foods have not been assessed for cariogenic potential, and a variety of different methods have been used for the assessment of other food-stuffs, including plaque acidity models (Edgar & Geddes, 1986), animal models and enamel demineralisation tests (Bibby et al., 1983). Difficulties can, therefore, be encountered in comparing the cariogenic potential of food-stuffs which have been analysed using different techniques. In addition, several items are frequently eaten together, and it has been shown that the sequence of intake of different foods can affect the plaque pH response (Geddes et al., 1977; Rugg-Gunn et al., 1981). This, together with the fact that it is difficult to assess intervals between each intake, especially at meal-times, means that results obtained with reference to subjects' diets must be interpreted with caution.

In this study, therefore, detailed analysis of diet was not attempted, and only an indication of the average intake frequency of potentially

cariogenic or potentially protective items was calculated, and a subjective assessment of a "typical day", for each subject, was made. Thus, one could only determine whether obvious differences were found amongst the individuals, and assess how the pattern of intake compared with the nine extra-oral sucrose applications being carried out in two of the three experimental protocols. While the analysis showed some variation amongst subjects in frequency of intake, and differences in the relative proportions of food and beverages with cariogenic potential, no relationship was apparent between the diets of these individuals, during the experimental periods, and the amounts of *in situ* demineralisation which developed.

4.6.3. Use of Selective Media.

Most epidemiological studies investigating the relationship between plaque microflora and dental caries have been concerned primarily with the proportions and isolation frequencies of the *Strep. mutans* and *Lactobacillus* spp. in relation to the condition of the underlying enamel (Hoerman *et al.*, 1972; Loesche *et al.*, 1975; Duchin & van Houte, 1978; Lang *et al.*, 1987).

Since these organisms are normally present in low proportion, and often constitute less than one percent of the total flora of caries-associated plaque (Bowden *et al.*, 1975; Mikkelsen & Poulsen, 1976), selective media are required for their detection in plaque samples. However, although such media are used in an attempt to achieve both maximal recovery of target organisms and complete selectivity regarding extraneous organisms, these ideals cannot be achieved at present, and the result is a compromise which, although not perfect, can still provide valuable information (Jordan, 1986).

The most commonly used selective medium for *Strep. mutans* is MSB agar, developed by Gold and co-workers in 1973. However, it is unable to support full growth of all *Strep. mutans* strains (Emilson & Bratthall, 1976; Little *et al.*, 1977), being partially inhibitory to a and b serotypes, which are not generally found in humans, and also to d/g serotypes which are present in 5 - 35 % of humans (Russell & Johnson, 1987). Another selective medium (TYCSB) was therefore developed by van Palenstein-

Helderman and co-workers (1983) in an attempt to improve recovery of Strep. mutans. Studies comparing the recoveries between the two media have shown differing results with some workers finding improved recovery with TYCSB (Matee et al., 1985), while Beighton (1986) found no significant difference between the mean counts of Strep. mutans isolated from TYCSB and MSB agar, but found more extraneous organisms on the former plates.

The selectivity of the medium normally used for the isolation of lactobacilli is based on the organisms' low pH, high acetate content and on the presence of a surfactant, Tween 80 (Rogosa et al., 1951). The medium selects for the whole spectrum of homofermentative and heterofermentative lactobacilli and, although it is thought they all have some cariogenic potential, some studies suggest that hetero-fermentative species are associated to a greater extent with high caries activity (Jordan, 1986).

In the present study, MSB and Rogosa SL were the selective media employed for the culture of Strep. mutans and Lactobacillus spp. respectively. The counts obtained from these media were expressed as a percentage of the total cultivable flora obtained from the non-selective blood agar plate. However, there are problems associated with comparing counts obtained from different media, particularly when one such as MSB agar is partially inhibitory to the organism. This was seen in the present study when Strep. mutans was isolated from both an MSB plate and blood agar culture from the same plaque sample. The count obtained from the blood agar plate frequently constituted a higher proportion of the total flora than the count calculated from the MSB culture. When this occurred the blood agar plate was used for the Strep. mutans count.

However, although one must be aware of the limitations of selective media usage, they allow information to be obtained about organisms which comprise only a small proportion of the plaque flora, and which would not normally be isolated if only non-selective media were employed.

4.6.4. Effect of Enamel Position on the Composition of the Plaque Microflora.

The side and position of the enamel sites under study has not generally been considered in investigations of the plaque microflora relationship to enamel demineralisation. However, the intra-oral appliance model used in the present study allowed these variables to be investigated, and analysis of the plaque microflora which was sampled from the left and right sides of the appliance, and the different positions within each trough area, showed these factors did not significantly affect the percentage distribution of organisms. This, therefore, allowed subsequent results to be tabulated for all positions combined within treatment groups.

4.6.5. Effect of Sequence of Experimental Runs.

A significant difference was found in the proportions of the predominant flora isolated from experimental runs one, two and three, for the seven individuals combined. This was, in part, due to the fact that the order of the runs was not balanced as, in six of the seven subjects, run one did not involve inoculation of Strep. mutans, this organism being isolated from the individuals' plaque samples at the end of the first run. Furthermore, Subjects F and G did not participate in the SPM protocol.

In addition, variation in the salivary counts of organisms, and the diets of the individuals during the different three-week experimental periods, probably also contributed to differences in the proportions of the flora at the different runs. The statistical analysis of variance used in this study, took into account the run effect when determining the relationship between flora, treatment condition and enamel demineralisation.

4.6.6. Effect of Treatment Conditions on Plaque Microflora.

The mean proportions, for all subjects, of the cultivable microflora of three week plaque, grown with no stressing, was similar in samples obtained from both sections and slabs. However, there was considerable inter-subject variation in the results, in particular in relation to Subject G, in whom slab plaque had a very low proportion of positive bacilli, and higher levels of positive and negative cocci than the other subjects. In

both section and slab plaque from Subject E, the level of Strep. mutans was higher than in the plaques from the other six subjects.

There are relatively few published reports of the microflora of smooth surface three week plaque, most plaques of this age being obtained from the gingival margins for periodontal research. Syed and Loesche (1978) sampled plaque from gingival inter-proximal areas, after 21 days of no oral hygiene. The mean values of the proportions of the groups of organisms isolated are similar to the results of the current study. Two week plaque removed from supra-gingival areas, which had received professional cleaning prior to the experimental period, contained a higher proportion of Gram negative rods and fewer positive cocci (van Palenstein Helderman, 1981). However, the results from the present study are within the ranges of percentage distribution of bacteria in supragingival mature plaque.

The microbial composition of three week plaque, grown under sucrose conditions, was very similar when collected from both sections and slabs. The proportions of the organisms were little different from those of unstressed plaque in situ with, in both cases, positive cocci and bacilli predominating, although the bacillary count was slightly higher. The application of sucrose had no significant effect on the mean proportion of Strep. mutans, which was less than 5 % in plaque obtained from slabs and sections, under both normal and sucrose plaque conditions. The isolation frequency and median proportion of Strep. mutans isolated from enamel sections were, however, slightly higher under SP conditions, while the values obtained from slabs were very similar in both treatment conditions. In plaque obtained from slabs, the proportions of Strep. oralis and negative bacilli were significantly higher in plaque which had been exposed to sucrose, compared to unstressed plaque.

No significant difference in total microbial count per unit area enamel surface was found between the normal and sucrose plaques, with the $\log_{10}$ count of the latter being slightly lower than that of the former. The counts of Strep. mutans and lactobacilli were also similar under both treatment conditions. This finding differs from that of Staat and co-workers (1975) who found increased numbers of viable microorganisms associated with sucrose-rich diets compared to the corresponding plaque

from low sucrose diets. However, comparison of normal and sucrose plaques is difficult as there is considerable variation in plaques obtained from different sites under the same conditions, and the normal diet of the subject will influence the degree of additional stressing resulting from the extra-oral sucrose applications (Bibby, 1976).

de Stoppelaar and co-workers (1970) have reported that when carbohydrate is excluded from the diet, the level of Strep. mutans falls to undetectable values, and the proportion of Strep. sanguis rises. When the subject is given sucrose again, the level of the former returns to, or exceeds, the subject's normal level of this organism. However, when Scheie and co-workers (1984) compared high and low sucrose diets they found little difference in the prevalence of Strep. mutans in three week plaque, with the organism persisting in low sucrose conditions. The Strep. mutans levels were low in plaque obtained during both dietary regimens in Scheie's study, whereas de Stoppelaar and co-workers included surfaces heavily infected with the organism. Although there was no increase in Strep. mutans prevalence in the study by Scheie and co-workers, the isolation frequency increased at discrete sampling sites in the sucrose rich diet, a finding similar to the results of the present study, where Strep. mutans was detected more frequently in plaque samples obtained from enamel section sites under SP conditions.

Another factor which must be considered is that in the current study, the subject's normal diet was maintained during experimental periods, including the "normal plaque" runs, whereas Scheie and co-workers and de Stoppelaar restricted the sucrose intake of their volunteers. Therefore it is perhaps not surprising that the difference between the microflora of unstressed plaque and the plaque obtained when sucrose was applied directly, was less than that described by these other workers.

The isolation frequency and mean proportion of Lactobacillus spp. was not affected by the additional sucrose exposure in some of the experimental runs. This finding differs from the results of Staat and co-workers (1975) who found increased levels of lactobacilli associated with high sucrose diets, and the difference between the studies is again probably due to the differences in background diets of individuals involved.

4.6.7. Implantation of Strep. mutans.

The inoculation of Strep. mutans on to the plaque caused a marked rise in the mean proportion of the organism isolated from both sections and slabs. However, this increase was almost entirely attributable to rises in Subjects A and E, the mean proportions in all other subjects remaining below 2 %, although the isolation frequencies in these subjects increased. The mean percentage of Lactobacillus spp. isolated was significantly higher in SPM plaque, for sections and slabs, compared to sites where inoculation of Strep. mutans was not performed. The mean proportion of Strep. sanguis was lower in both SPM plaques compared to NP and SP.

Variation in the ability to implant successfully Strep. mutans strains in the human oral cavity has been found by some workers (Svanberg & Krasse, 1981), and others have found great difficulty in implanting Strep. mutans on to natural tooth surface in any individual (Krasse et al., 1967; Jordan et al., 1972; Svanberg & Loesche, 1978a). It has been suggested that the failure to establish Strep. mutans in these studies may have been due to the coating of the cells with non-human proteins from culture broths, as the cells were not suspended in salivary components pre-implantation (Svanberg & Krasse, 1981). Even variation in salivary composition between subjects may be important, since it has been shown that Strep. mutans cells treated with saliva from subjects with endogenously high Strep. mutans counts, implant more successfully in volunteers than do cells treated with saliva from subjects with naturally low Strep. mutans salivary counts (Svanberg & Krasse, 1981).

However, the coating of cells in saliva may reduce the potential of Strep mutans to adhere to the pellicle or plaque-covered surfaces, since the bacteria may combine with salivary components, covering the binding sites or causing agglutination of the cells (Gibbons, 1984).

In the present study, each subject's own Strep. mutans cells were implanted on to the enamel test areas suspended in the subject's own clarified saliva. This procedure was carried out four times during the first 28 hours of the experiment, to increase the likelihood of successful implantation. The first application was on to the dry enamel specimens, before the appliance had been placed in the mouth, the saliva in the

suspension forming a coating on the enamel surface, to which the Strep. mutans cells could adhere (Gibbons et al., 1986). The implantation procedure was repeated after four hours' oral exposure as, by then, the enamel surfaces would be covered with a pellicle, to the surface of which, pioneer species adhere. The two final applications of cells were performed when the early microbial flora was established, and production of extra-cellular polysaccharides, from the extra-orally applied sucrose, may have commenced. Thus, the Strep. mutans may have had the opportunity at this time to adhere either to the other bacteria present in the plaque, or to the glucan in the matrix.

The concentration of Strep. mutans cells implanted on to the test sites in each subject was similar, and the isolation frequencies and absolute counts of the organism per mm^2 of enamel slab surface was higher in SPM plaque in all subjects compared to normal and sucrose plaques. However, the increase in the proportion of Strep. mutans in the total count was appreciable in only two of the five subjects.

Factors which are thought to influence the ability of Strep. mutans to implant successfully include the hydrophobicity of the cells (Svanberg et al., 1984), the diet and salivary characteristics, including Strep. mutans count, and the normal plaque flora of the individual (Svanberg & Krasse, 1981). In the current study, of the two individuals where there was a marked rise in Strep. mutans following implantation, one had high natural salivary and plaque levels of the organism, while the other had the lowest natural Strep. mutans salivary count of the five subjects, but did, however, have poor salivary flow rate and buffering capacity. In addition, this subject had the lowest total microbial counts under normal plaque conditions. This finding is in agreement with Svanberg and Krasse (1981), who found that subjects with the lowest total colony forming unit counts in unstressed plaque had the best implantation of Strep. mutans.

In the individual where there was good implantation of Strep. mutans and a low natural plaque and salivary count of this organism, no increase in proportion or isolation frequency of Strep. mutans occurred in the normal or sucrose plaque on the one side of the appliance, when Strep. mutans was implanted on to the opposite side. Similar results were reported by Svanberg and Loesche (1978b), who found that following implantation of

Strep. mutans into artificial fissures, the organism became established only on the ipsilateral side. These authors suggested that the local establishment was probably due to direct contact between teeth, and the shedding of viable cells in relatively high concentration into saliva locally. Dilution of the organism in the saliva would make establishment at distant sites less likely.

4.6.8. Effect of Treatment Condition on Demineralisation.

Few studies have been undertaken involving the assessment of mineral loss from enamel sections, as most intra-oral demineralisation studies have used the natural dentition, or enamel slabs mounted on an appliance. Short-term investigations of early carious lesions have involved visual assessment of natural tooth surfaces, following no oral hygiene and frequent sucrose application, either to the whole dentition (von der Fehr et al., 1970; Geddes et al., 1978), or to isolated surfaces in the mouth (Scheie et al., 1984). However, accurate quantification of the white spot lesions produced in such studies is not possible.

In the current investigation, the enamel "single section" technique was used, as it allows accurate quantification of mineral content pre- and post-experimentally. Thus the demineralisation occurring during the experimental period can be calculated.

The densitometric profile of the enamel lesions produced in this study were similar to the mineral distribution profiles of early carious lesions observed in in vitro and in vivo studies (Arends and Christoffersen, 1986). The sites showing surface softening, without a surface layer represent the initial stages of enamel demineralisation, while the more advanced lesions showed evidence of a surface layer with subsurface demineralisation.

The mean amount of demineralisation found in sections which had been exposed to normal plaque was 275 % vol min x μm, with intra-subject variation being seen, as well as differences between subjects. Dijkman and Arends (1988) used microradiography to assess mineral loss from enamel slabs, after intra-oral demineralisation experiments. They reported a mean total mineral loss of 719 ± 246 vol % x μm, from human

enamel after a four week experimental period, during which no additional stressing was employed.

These results are much higher than the mean Δz values obtained in the present study from both NP and SP conditions, and are even slightly higher than the SPM conditions. However, the duration of the experiment using enamel blocks was seven days longer than the current studies, and the Δz values quoted by Dijkman and Arends represent the change in mineralisation as measured post-experimentally only, as the mineral loss could not be measured pre-experimentally. In the present study, the Δz value was calculated by subtracting the baseline measurement of mineral loss from the final demineralisation value. Therefore, it would be expected that these results would be lower than the findings reported by Dijkman and Arends. Furthermore, the latter study involved 12 volunteers, the details of whose salivary and plaque counts of Strep. mutans, diet and caries experience are not known, so a direct comparison with the present study is not possible.

In the current study, stressing the plaque by the application of sucrose resulted in an increase in the mean amount of demineralisation, compared to the mean value for the mineral loss following normal plaque conditions. This agrees with findings of other workers, where an overall increase in demineralisation is seen on the natural dentition, with intra-oral sucrose rinses (von der Fehr et al., 1970; Geddes et al., 1978; Scheie et al., 1984), and on enamel blocks mounted on an intra-oral appliance and exposed to extra-oral sucrose applications (Ostrom & Koulourides, 1976; Gallagher & Pearce, 1979). As found in the present study, variation in the amount of demineralisation was noted on repeat runs on the same individual and inter-subject variation also occurred (Ostrom & Koulourides, 1976).

A significantly higher level of demineralisation was seen, in the present study, in sites which received implantation of Strep. mutans and extra-oral sucrose applications, compared to NP and SP conditions.

Borden and co-workers (1980) seeded Strep. mutans cells on to enamel slabs which were covered with Dacron mesh and mounted on an intra-oral appliance. The Strep. mutans strain implanted in each individual's

appliance had been isolated from that subject's flora, and rendered Streptomycin resistant, to allow identification of the implanted strain in the plaque microflora. The seeding procedure involved 2-hour in vitro inoculation of the organism with the gauze covered slabs which had been mounted on the appliance. This study involved four volunteers, and found that successful implantation of Strep. mutans occurred in every subject, with the mean count after seven days of appliance wear comprising 58 % of total flora, compared with approximately 1 % on the control side. There was considerable inter-subject variation regarding demineralisation levels but, unlike the present study, no overall significant increase in demineralisation was seen in sites which had been inoculated with Strep. mutans. The authors suggested that the high Strep. mutans counts obtained were due to entrapment of the organism in the Dacron mesh rather than adhesion of the cells to the tooth surface, and so the natural environmental conditions did not select for the increase. In the present study, the enamel sites were left uncovered, hence the increase in proportion and isolation frequencies of Strep. mutans following implantation indicated they had been able to compete successfully, with other plaque organisms, but to varying extents in different subjects, with the local environment selecting for the continuing presence of Strep. mutans in the plaque.

The strains of Strep. mutans seeded on to the mesh covered enamel surfaces by Borden and co-workers (1980) may not have been as cariogenic as other strains, and the authors also emphasise that other plaque organisms are also capable of high acid production and that many other factors such as saliva, diet and fluoride also play an important role in determining caries susceptibility.

4.6.9. Relationship Between Plaque Microflora and Demineralisation.

In view of the problems known to affect cross-sectional and longitudinal studies on the microflora of plaque associated with caries, as discussed in Chapter I, this in situ appliance study was designed to allow small plaque samples to be removed from discrete enamel locations, thus permitting the compostition of the plaque microflora to be related to changes in the underlying enamel. However, although small enamel windows, measuring

1 - 2 mm, were used, this method does not allow absolute correlation between plaque microflora and enamel surface, due to the possible presence of microcolonies of specific organisms that may have been associated with minute areas of demineralisation but which would account for only a small proportion of the total plaque microflora.

As enamel sections yield only very small quantities of plaque, which cannot be weighed accurately, the experiments also utilised enamel slabs, which allowed the plaque microflora to be expressed as absolute counts of bacteria per mm^2 enamel surface rather than simply as proportions of the total microflora, as is the case with the sections. Pre-experimental quantification of mineral content of enamel slabs was not possible, as described previously (Section 1.12.7.). However, attempts were made to assess demineralisation post-experimentally, but as the maximum thickness of the slab which allowed them to be accommodated in the lingual flanges of the appliance was 1 mm, the slabs were brittle, and liable to fracture during attempts to section them. Thus, only the enamel sections were available for studying the relationship between plaque microflora and demineralisation of the underlying enamel.

Strep. mutans in Relation to Demineralisation.

The results showed that under normal plaque conditions, the isolation frequency of Strep. mutans increased significantly with increasing mineral loss. The mean proportion of Strep. mutans was 1.8 %, 5.4 % and 3.9 % in Δz Groups 1 to 3, respectively, but the range of the proportions in each group was large, so the mean percentage counts were unduly weighted by a few results which were very much higher than the others. Therefore, the medians of the proportions probably provide a better indication of the trend of the values than do the means. While the means showed no pattern with increasing demineralisation, the median was higher in Δz Groups 2 and 3, at 0.1 %, compared to Group 1 where it was Not Detectable.

Similarly, in plaques exposed to sucrose nine times daily, the isolation frequency of Strep. mutans increased with increasing demineralisation. The organism was detected in 62 % of sites with mineral loss below 200 units, and in every site where demineralisation exceeded 1,000 units.

Large variation in Strep. mutans counts within each Δz group again occurred, with the means showing no trend with increasing mineral loss, although the median of the proportions was 0.1 % for each of Δz Groups 1 to 3, and 1.8 % for Δz Group 4.

For NP and SP conditions combined, there were eight sites in which plaque Strep. mutans comprised more than 10 % of the total cultivable microflora, and each was obtained from Subject E. The distribution of these sites showed no obvious pattern, with the percentage of sites fitting this criterion being 4, 18 and 10 % in NP Groups 1 - 3, respectively, and the percentages for SP Groups 1 to 4 being 12, 0, 12 and 0 %. Thus, the highest levels of Strep. mutans were not associated with the enamel sites where the greatest amount of demineralisation occurred.

Under SPM conditions, the mean proportion of Strep. mutans increased with increasing mineral loss, ranging from 8.9 % in Δz Group 1 to 30.5 % in Group 4. Although the mean percentages were fairly similar for Δz Groups 2 to 4, comparison of the medians demonstrated a significantly higher level in Group 4, compared to the other three groups. The isolation frequency of the organism was high in every demineralisation group, and as with sucrose plaque, Strep. mutans was isolated from every site where demineralisation exceeded 1,000 units.

Isolation Frequency of Strep. mutans.

The findings in the present study of an increase in isolation frequency of Strep. mutans with increasing mineral loss, is in agreement with many cross-sectional and longitudinal studies which have shown that Strep. mutans is isolated more often from carious sites than from caries-free surfaces (Hoerman et al., 1972; Loesche et al., 1975; Duchin & van Houte, 1978; Lang et al., 1987).

However, in the current study, Strep. mutans was also isolated from many sites in which minimal demineralisation occurred. Most cross-sectional studies have also found Strep. mutans associated with clinically and radiographically sound sites (Hoerman et al., 1972; Loesche et al., 1975; Mikkelsen et al., 1981) and the study by Mikkelsen and co-workers found Strep. mutans present on almost every site, whether or not

demineralisation was present, in a group of subjects with high caries activity. Milnes and Bowden (1985) found that this organism was isolated from caries susceptible sites, irrespective of the presence or absence of caries at an individual site.

Longitudinal studies tend to show an increase in isolation frequency of Strep. mutans associated with detection of a lesion (Hardie et al., 1977; Kristoffersson et al., 1985; Lang et al., 1987). However, the detection of an increase in isolation frequency immediately prior to the detection of a lesion is more variable, with some studies showing no increase at this stage (Hardie et al., 1977), whilst others show an increase in the months prior to the detection of a lesion (Ikeda et al., 1973; Keene & Shklair, 1974; Loesche & Straffon, 1979; Walter, 1982).

Longitudinal studies also find sites which remain caries-free throughout the experimental period, although they are associated with plaque containing Strep. mutans. In a two year study by Kristofferssen and co-workers (1985), 48 % of the Strep. mutans positive sites fitted into this category.

The presence of Strep. mutans, sometimes even in high numbers, does not necessarily indicate, therefore, that caries has occurred or imply that it will occur in the near future. However, with clinical studies, some demineralisation may have occurred, but to such a limited extent as to be undetectable. As an increase in the isolation frequency of Strep. mutans has been found to predate the development of a lesion, it is possible that, in the present study, lesions would have developed on the sites associated with Strep. mutans, had the duration of the experiment been longer.

In the current study, Strep. mutans was isolated from every site which experienced demineralisation of over 1,000 % vol min x μm during the three week experimental period, but the organism was not detected in some of the samples where the mineral loss was between 500 and 1,000 Δz units. This finding is similar to other reports which show that most carious sites are associated with Strep. mutans but that some lesions are found where the overlying plaque is apparently free of this organism (Hoerman et al., 1972; Hardie et al., 1977; Kristoffersson et al., 1985; Lang et al., 1987).

The development of lesions in apparently Strep. mutans-free sites may be due to their presence in low numbers, which would not be detected due to inappropriate sampling methods or to inadequate laboratory techniques. For example, if a relatively large plaque sample is removed from an area in which there is a small discrete site of demineralisation, specific odontopathogenic organisms may be concentrated at the lesion, but diluted in the large plaque mass and therefore undetectable on sampling (Duchin & van Houte, 1978). The cultural methods may also be responsible for the non-detection of these organisms because, as discussed in Section 4.6.3., even the use of selective media has limitations (Jordan, 1986). A number of workers (Hardie et al., 1977; Loesche & Straffon 1979; Lang et al., 1987), have also suggested that other members of the plaque flora, such as acid-producing lactobacilli or peptostreptococci (Bowden et al., 1984) may either alone, or in combination with proportions of Strep. mutans below detection limits, initiate the demineralisation process.

Counts of Strep. mutans.

The overall results, with the three treatment conditions combined, showed an increase in the mean and median percentage counts of Strep. mutans with increasing demineralisation, with the mean ranging from 5.2 % in Δz Group 1 to 20.0 % in Group 4. This finding, however, was largely attributable to the results obtained under SPM conditions where a corresponding increase from 8.9 % to 30.5 % occurred.

In the normal plaque in this study, the median values of Strep. mutans were ND in Δz Group 1, and 0.1 % in samples where the lesion was greater than 200 units. In SP plaques, the median count was 0.1 % for all Δz Groups representing lesions of less than 1,000 units, but 1.8 % where the mineral loss exceeded this value. Thus, the results for NP and SP lie within the range of results quoted by other authors, where Strep. mutans associated with caries often constitutes less than 1 % of the total flora (Bowden et al., 1975; Mikkelsen & Poulsen, 1976; Duchin & van Houte, 1978).

Duchin and van Houte (1978) sampled the plaque overlying smooth surface incipient lesions and the surrounding sound enamel from 6 - 12 year old children with multiple decayed teeth, and found the mean and median

values of the Strep. mutans proportions for the carious sites to be 7.5 and 1.4 % respectively, with the corresponding values from sound surfaces being 0.5 % and 0.02 %.

A study on children under 10 years, by Loesche and co-workers (1975), also found a significantly higher mean level of Strep. mutans associated with carious fissures (18.7 %) compared to caries-free fissure sites (7.3 %). Both these values are much higher than the NP and SP results in the current study, but are more in keeping with the proportions obtained from plaques under SPM conditions. The diagnostic criterion for carious sites in the Loesche study was based on the presence or absence of surface cavitation, and therefore represented a very advanced stage in the disease process.

A study by Mikkelsen and co-workers (1981), on an adult population with high caries prevalence, also found high proportions of Strep. mutans in plaque samples from approximal sites, but unlike previous studies found higher mean and median values in plaque from caries-free surfaces (7.6 and 4.1 % respectively) compared to carious areas (mean 6.3 %, median 0.7 %). Similarly, in a study involving young men aged 17 - 19 years, Hoerman and co-workers (1972) found that the Strep. mutans count, given as a mean percentage of the total streptococcal count, was slightly higher in plaque obtained from radiographically sound sites, compared to plaque from carious sites. In this study, no median values were reported.

The results obtained from longitudinal studies regarding the relationship between Strep. mutans proportions and the initiation of caries are also equivocal. Many studies have shown an increase in Strep. mutans levels prior to the detection of, and during the development of, lesions on fissures and smooth surfaces (Ikeda et al., 1973; Loesche & Straffon, 1979; Lang et al., 1987), while others have found no significant relationship between Strep. mutans levels and the initiation of caries, with the rise in Strep. mutans count only dating from the time of detection of the lesion (Mikkelsen & Poulsen, 1976; Hardie et al., 1977). However, it is considered that the isolation frequency and mean proportion of Strep. mutans are generally higher in plaque obtained from carious tooth surfaces, compared to sound sites (van Houte, 1980). Similar results were found in the present study.

Lactobacillus spp. in Relation to Demineralisation.

After Strep. mutans, lactobacilli are the organisms which have received the most attention with regard to the microbial aetiology of dental caries.

In the present study, lactobacilli were detected in the plaque from at least 50 % of the enamel sites in each Δz Group, and under each treatment condition, but no relationship between isolation frequency and level of demineralisation was seen. The median values from sites experiencing mineral loss of under 500 μm was 0.1 % or less with all treatment conditions, with an increase to 1.6 % (SP) and 2.8 % (SPM) in plaques associated with demineralisation of between 500 and 1,000 Δz units. The range in the Lactobacillus spp. proportion of the total cultivable microflora was large, with some sites which experienced minimal changes in mineral content being associated with plaques in which lactobacilli comprised as much as 41.4 % of the total flora. This differs from other studies which have shown that sound surfaces are very rarely associated with high lactobacillary levels (van Houte, 1980).

The low levels of lactobacilli associated with demineralisation below 1,000 Δz units is in agreement with the findings of many epidemiological studies in which this organism was not detectable, or present in very low proportion, on sound enamel surfaces (Duchin & van Houte, 1978), and does not increase in isolation frequency or proportion to any marked extent before the diagnosis of caries (Ikeda et al., 1973; Hardie et al., 1977). However, a few studies have shown a significant increase in the proportion of lactobacilli 6 - 12 months prior to the diagnosis of caries (Loesche et al., 1984).

The isolation frequency of Lactobacillus spp. in the present study was somewhat higher than has been reported on other caries-free or incipient caries sites (Hardie et al., 1977; Duchin & van Houte, 1978), and this may have been due to the greater retentiveness of the sites on the appliance model. Lactobacilli appear to have a relatively low affinity for natural tooth surfaces, while appliances such as orthodontic bands and dentures have been shown to increase dramatically the numbers of these organisms in the mouth (van Houte et al., 1972). Milnes and Bowden (1985) found significantly higher isolation frequencies and levels of

Lactobacillus spp. in caries-susceptible sound enamel sites compared to non-susceptible control sites.

A highly significant increase in Lactobacillus spp. mean and median proportions was seen, in the present study, with demineralisation of over 1,000 Δz units, compared to plaque associated with lower levels of mineral loss. This finding is in agreement with many studies which have found a marked increase in lactobacilli only after the detection of a lesion (Ikeda et al., 1973; Loesche & Straffon, 1979). However, in some epidemiological studies, caries surfaces have been found where lactobacilli were not isolated from the overlying plaque (Ikeda et al., 1973; Duchin & van Houte, 1978), and Hardie and co-workers (1977) found no significant increase in Lactobacillus spp. proportion following caries detection. In the current study, a few sites were found in which demineralisation of over 1,000 Δz units had occurred, with no or very low proportions of lactobacilli being isolated from the associated plaque samples.

The Lactobacillus spp. mean (18.0 %) and median (12.5 %) proportions associated with Δz Group 4 in this study were extremely high. These results were obtained from the sites inoculated with Strep. mutans and receiving extra-oral sucrose applications. The acid environment created by this combination, together with the retentive nature of the appliance, was probably responsible for the selection of lactobacilli in the plaque samples.

In this current work, the Lactobacillus spp., L. casei, L. acidophilus, L. brevis, L. fermentum and L. plantarum were all isolated on occasions from plaque samples overlying the enamel sites, with L. casei and L. acidophilus being found most frequently. Some studies investigating the relationship of plaque microflora to caries in humans have not identified the lactobacilli associated with lesions, to species level (Loesche & Straffon, 1979; van Houte et al., 1982), while Milnes and Bowden (1985) did so, and related their findings to cariogenicity testing of the species in animal models (Fitzgerald et al., 1980; 1981). Using the same criteria, all species identified in the present study have cariogenic potential, although perhaps to varying degrees (Jordan, 1986).

Of the 13 enamel sites associated with demineralisation of over 1,000 % vol min x μm, L. acidophilus, L. casei and L. fermentum were the species with the highest prevalence and isolation frequency, with L. fermentum comprising over 10 % of the total cultivable microflora, on six occasions.

Loesche and Straffon (1979) found some carious fissure sites which had undetectable or low levels of Strep. mutans, but high levels of Lactobacillus spp. In the present study, Strep. mutans was isolated from every Δz Group 4 lesion, but of the five sites where it comprised less than 1.5 % of the total flora, only two were associated with high Lactobacillus spp. levels (> 0.1 %).

This study tends to support the view that plaque Lactobacillus spp. levels increase in the presence of established demineralisation, as changes in the local environment select for this organism, which may then be associated with progression of the lesion through the enamel (Boyer & Bowden, 1985). Its role in the initiation of caries in this study remains unclear, however, since it was present in low proportion at most sites with small amounts of demineralisation, and increased slightly with mineral loss of between 500 and 1,000 Δz units.

Relationship of Lactobacillus spp. and Strep. mutans to Lesion Profile.

Higher proportions of Lactobacillus spp. were found in lesions showing obvious surface softening (8.4 %) and subsurface demineralisation (8.6 %), compared to sites showing no apparent change in lesion profile or only slight surface softening. This is in agreement with the results obtained from the Δz Groups, and again suggests that this organism tends to be associated with more extensive enamel demineralisation. Similar results were obtained regarding the mean proportions of Strep. mutans and lesion profile groups. With both organisms, the highest median values of the proportions were obtained from plaque associated with obvious surface softening of the enamel surface. Tooth surface defects, such as carious lesions, are thought to favour the retention of these organisms (van Houte, 1980) and the higher median levels found in Lesion Profile Group 3 may therefore have resulted from increased retentiveness of these sites, due to loss of mineral at the enamel surface.

The Role of Other Bacteria in Relation to Demineralisation.

Plaque bacteria other than Strep. mutans and lactobacilli have been investigated, to some extent, regarding their relationship with dental caries. Strep. sanguis has often been reported as being a prominent member of the plaque flora, but available data suggests that it is not positively correlated with caries activity, and in fact an inverse relationship between its level and the initiation and progression of caries has been found (Bowden et al., 1976; Loesche & Straffon, 1979; Loesche et al., 1984). Many studies show an inverse ratio between Strep. mutans and Strep. sanguis in relation to caries activity (Loesche & Straffon, 1979; Loesche et al., 1984).

With the changes in nomenclature of the streptococcal species, it is difficult to compare directly the results from different workers. In the present study, an overall slight decrease in the levels of organisms classified as Strep. sanguis and Strep. oralis was seen with increasing amounts of demineralisation, although a positive trend was observed between Strep. sanguis and mineral loss under SP conditions.

Strep. salivarius is normally present in only very low proportions in plaque, with the tongue being its primary habitat (Ostrom et al., 1977). The results of the relationship between this organism and caries activity are equivocal (van Houte, 1980). In the current study, the mean levels of Strep. salivarius comprised less than 5 % of the total cultivable flora in each demineralisation group, and showed a slight decrease as increasing mineral loss occurred.

Actinomyces spp. made up a large proportion of the three week plaque microflora, with most of the organisms belonging to A. odontolyticus and catalase positive and negative A. viscosus / naeslundii. The levels of Actinomyces, at the genus level, were relatively stable over the four Δz demineralisation groups. This result is similar to that of Hardie and co-workers (1977) who found no obvious difference in levels between carious and non-carious sites, while a recent cross-sectional study by Marsh and co-workers (1988) found higher counts at carious (73 %) compared to caries-free sites (50 %). However, as identification of Actinomyces spp. to species level was limited, in the current study, by the non-availability

of sophisticated techniques, it is possible that, had such methods of identification been employed, a correlation between the proportion of one or more of the species and the level of demineralisaton may have been found.

In this study, an overall slight decrease in Veillonella spp. was seen with increasing mineral loss, the decrease being most marked under normal plaque and SPM conditions. This has been found by other workers, and it has been suggested that as these organisms consume the lactic acid produced by other organisms, the absence of Veillonella spp. may reduce the potential for decreasing the caries risk in an individual (Bowden et al., 1976). However, other investigations have found an increase in proportion of Veillonella spp. in the presence of caries, and it has been suggested that the presence of the organism reflects the acid environment of the plaque which selects for this bacterium (Milnes & Bowden, 1985).

Other organisms such as Neisseria, Bifidobacterium, Propionibacterium, Eubacterium, Bacteroides, Capnocytophaga and Fusobacterium spp. were isolated on occasions from plaque samples overlying the enamel sites. With the exception of Bacteroides spp., which showed no significant relationship with amount of demineralisation, their infrequent isolation meant that no statistical analysis could be carried out. However, no obvious trend was apparent in relation to mineral loss. Very little attention has been paid to these organisms in human caries studies, because of the low counts normally involved. However, Strep. mutans and Lactobacillus spp. are often only present in very low proportions, and selective media are used to determine their isolation frequencies. Therefore, although animal and in vitro experiments suggest that Strep. mutans and lactobacilli are strongly implicated in the carious process, the involvement of these other organisms in relation to human caries cannot at present be ruled out, since comprehensive studies on them, using selective media, have not been performed.

Many studies have found a few carious sites where neither Strep. mutans nor Lactobacillus spp. has been detected in the associated plaque. Similarly, in the present study, a few enamel sites which experienced mineral loss of between 500 and 1,000 Δz units, were associated with plaque from which neither Strep. mutans nor lactobacillus was isolated.

This may be due in part to limitations in the detection techniques available for the isolation of these organisms, as mentioned previously (Section 4.6.3.), but also suggests that other organisms present in the plaque microflora may, either singly or in combination, be capable of producing demineralisation.

4.6.10. Cariogenicity Tests.

Only a relatively small number of experimental caries models have attempted to relate the composition of the plaque microflora overlying an enamel site to its change in mineral content. A seven day study by Ostrom and co-workers (1977), using bovine enamel slabs covered with a Dacron mesh and mounted on an intra-oral appliance, found that the highest demineralisation scores, as measured by microhardness testing, were associated with increased plaque proportions of Lactobacillus spp. and Strep. salivarius, while no correlation with Strep. mutans was seen. Extra-oral sucrose applications were employed by Ostrom and co-workers, and a similar appliance model was used by Gallagher and Pearce (1979). The latter found no consistent pattern in the counts of individual organisms, including Lactobacillus spp. and Strep. mutans, isolated from sucrose plaque which was associated with demineralisation, compared to the microflora of plaque from control sites. Both these studies were limited in their ability to relate plaque microbial composition to demineralisation, as they involved the use of Dacron gauze, the problems associated with this having been outlined previously. In addition, limited microbial characterisation of the plaques was carried out, with identification of streptococcal species and lactobacilli being performed only by observation of colonial morphology on selective media.

An intra-oral human caries model, involving the banding of teeth which were later to be extracted for orthodontic reasons, was able to produce white spot lesions on enamel under the orthodontic band within a four week period (Arneberg et al., 1984). A rapid increase in Strep. mutans and Lactobacillus spp. counts was found during the experimental period, suggesting a selection for these organisms in the localised acidic environment during early caries development. Unlike the study of Ostrom and co-workers (1977), who found that Strep. salivarius was prominent in

their caries model, this organism was not a predominant member of the microflora in the current study, nor in the Arneberg study.

Although many recent studies have involved the implantation of Strep. mutans on to enamel blocks mounted on an intraoral appliance, for short-term cariogenicity tests of sugars and foodstuffs, very few have monitored the composition of the plaque microflora on the blocks for any length of time following implantation, and related it to the degree of mineral loss. Many authors have inoculated Strep. mutans into fissures and monitored the success of colonisation of the area, but again they do not appear to have related it to changes in mineral content. Borden and co-workers (1980), who implanted Strep. mutans on to Dacron-covered enamel found higher levels of Strep. mutans following implantation, but no difference in demineralisation between test and control sites. The possible reasons for these findings and comparison with the current study have already been discussed (Section 4.6.8.).

4.6.11. Effect of Abrasion of Enamel Surface on Plaque Microflora and Demineralisation.

As removal of the outer layer of enamel, with high but irregular amounts of fluoride, may lead to a more uniform and more susceptible enamel surface (de Groot et al., 1986), an increasing number of intra-oral and in vitro demineralisation studies have been carried out using abraded enamel in an attempt to produce more rapid and extensive demineralisation, with less variability between different sites. However, it appears that no study has investigated whether the treatment of the enamel surface in this manner affects the development of the overlying plaque microflora. This current study, therefore, compared both the degree of demineralisation and the microbial composition of plaque associated with non-abraded and abraded enamel surfaces.

The proportions of the predominant organisms isolated from the enamel sites showed some variation within each enamel surface and plaque treatment condition group. However, no significant difference in either the percentage composition of predominant microflora or the absolute counts per mm^2 enamel surface was found between the abraded and non-abraded enamel surfaces. This, therefore, suggests that the abrasion of

the surface enamel does not affect the development of associated plaque flora, and that microbial considerations do not argue against its use in intra-oral studies.

This investigation was carried out on Subject A, and, as was found in the main demineralisation study, the implantation of Strep. mutans together with frequent extra-oral sucrose applications, resulted in significantly higher proportions and absolute counts of this organism in three week plaque samples, compared with normal plaque. However, in this case, only a very slight mean increase in demineralisation of non-abraded enamel sites occurred in the SPM compared to the NP conditions. Similar levels of mineral loss occurred following exposure of the abraded enamel surfaces to normal plaque conditions, but the combination of SPM conditions and abrasion of the enamel surface resulted in significantly higher mean levels of demineralisation than in the other three groups. However, a very large range in Δz values, from 0 to 4598 units, was seen.

The isolation frequency of Strep. mutans increased with increasing demineralisation in the abraded enamel sites, and the mean and median proportions were highest in Δz Group 4. However, some abraded sites which experienced minimal mineral loss were associated with plaques in which Strep. mutans comprised as much as 69 % of the total flora, while in two other sites where demineralisation of between 500 and 1,000 Δz units occurred, the levels of Strep. mutans were 0.1 and 0.3 %. The median value for Lactobacillus spp. was Not Detected in each Δz group, and it was isolated from only 30 % of enamel sites where mineral loss exceeded 200 units.

Thus, while abrasion of the enamel surface resulted in significantly higher amounts of demineralisation overall, a very large range in mineral loss values was seen, which could not be explained by the relative levels of Strep. mutans or lactobacilli in the overlying plaque. This finding is in agreement with the in vitro results presented in Chapter III, which found that abrasion of the enamel surface resulted in significantly greater mineral loss, following a standard acid attack, but that the variation in demineralisation of enamel was not reduced by removing the outermost layers of the surface.

The variation in the degrees of demineralisation seen in this in situ study was due to a combination of difference in susceptibility of the abraded enamel sites, and the many other intra-oral factors involved in the carious process. While the amount of demineralisation increased following removal of the outer layers of enamel, abrasion did not seem to affect the composition of the overlying plaque flora, suggesting that it would be suitable for use in intra-oral demineralisation studies. These results also show that abrasion of enamel does not appear to reduce the problems associated with variation in susceptibility of different enamel sites to demineralisation.

4.7. Conclusions.

In this in situ appliance study, potential causes of conflict were standardised as far as possible, and the effect of such factors as side of mouth, position on appliance and order of treatment runs were assessed. Statistical analysis of variance of the results took into account the effect of these factors, when determining whether there were any associations between treatment conditions, microflora and demineralisation.

Considerable inter-subject variation was found, in common with other studies, regarding composition of the microflora and levels of demineralisation. In a study of this size, such findings are inevitable, but while the use of many more subjects would help to overcome this problem, this is not practicable because of the large number of plaque samples and enamel sites which would require analyses. Inter-subject variation was again taken into account by the method of statistical analysis employed.

Stressing of the plaque with extra-oral sucrose applications was found to have no significant effect on the plaque microflora, with regard to its percentage composition and total bacterial counts per unit area of enamel. However, the additional stressing resulting from the frequent sucrose applications, in addition to the subject's normal diet, resulted in slightly higher levels of demineralisation than were found with the protocol where no sucrose applications were employed.

The inoculation of the subject's own Strep. mutans, in addition to sucrose stressing, produced an increase in isolation frequency and mean proportion

of Strep. mutans in each subject, but inter-subject variation was very marked. These findings were in accordance with the results of previous studies. The above protocol also resulted in an increase in the levels of lactobacilli isolated from the enamel sections, and was associated with significantly higher levels of demineralisation.

The demineralisation seen in this study was typical of early caries lesions, with either surface softening, or sub-surface demineralisation, being found.

Pre-experimental abrasion of the enamel surface was associated with an increase in mineral loss, but had no effect on the composition of the overlying plaque flora. However, the variability in demineralisation between sites was as great as the variability between non-abraded sites. Thus, although abrasion may be of value in producing larger lesions in a short-term study, the problem of variation in susceptibility between sites has not been overcome.

An increase in the isolation frequency of Strep. mutans, as well as its mean and median proportional counts, was seen in association with higher levels of demineralisation. The isolation frequency of Lactobacillus spp. was higher than in many epidemiological studies, and this may have resulted from the increased retentiveness of the appliance model. Highly significant increases in mean and median counts were seen in lesions greater than 1,000 % vol min x μm, and this agrees with other studies, which have found that lactobacillary levels increase markedly, only following detection of a lesion.

While these organisms, particularly Strep. mutans, seem to be associated with enamel demineralisation, many sites were found in which high levels of the streptococcus were present in the absence of a lesion. There were, in addition, a few sites where mineral loss of between 500 and 1,000 units occurred, but neither Strep. mutans nor Lactobacillus spp. was isolated. This suggests that other factors, such as other salivary characteristics, host diet and enamel susceptibility, must be involved in caries development, and may modify the attack of cariogenic organisms. The presence of demineralisation in the absence of detectable Strep. mutans and Lactobacillus spp. suggests that any of the other acid-producing organisms present in the plaque may have been responsible for

the development of the lesion. However, Strep. sanguis and Strep. oralis decreased slightly with increasing mineral loss, and Actinomyces spp. showed no trend in relation to demineralisation.

It has been suggested that, in sites where demineralisation does not occur in the presence of high levels of Strep. mutans, this may be due to other organisms consuming the acid produced. Veillonella spp. have been implicated in this theory, but in this study, the level of the organism was not particularly high in the caries-free sites where Strep. mutans was abundant. Veillonella spp. were found to decrease with increasing demineralisation, and were thus not selected for by the acid environment. However, it may be that the lack of Veillonella spp. to consume the acid resulted in the larger lesions at these sites.

In this study, while there was a trend towards an association between salivary characteristics and experimental mineral loss, the only subject characteristic which was a significant indicator of the amount of demineralisation in the test sites, was the DMFS score of the subject.

In most studies, the microbiological results are expressed as proportional, rather than absolute counts. This method is adopted because of the small samples involved, but has limitations, in that comparison between results is difficult, since a given proportion of Strep. mutans, in different samples, may represent vastly different numbers of the organism, as can be seen from a comparison of the mean proportions and absolute counts, in plaque obtained from enamel slabs.

Many problems are experienced in relating plaque microflora to mineral loss, including identification techniques and the fact that many intra-oral factors such as salivary characteristics, diet and enamel susceptibility, play a role in the demineralisation process. However, the microbiological findings in relation to demineralisation in this study are similar to the results of the cross-sectional and longitudinal investigations which have been performed. In addition, the use of the intra-oral appliance has several advantages over such investigations.

The appliance allows short-time studies to be undertaken, with cariogenic stressing of the test sites without affecting the natural dentition. As

demineralisation can be produced at many sites within the time-scale of the experiment, fewer sites need be employed, compared with epidemiological investigations, where the percentage of carious sites, in the total number of sites studied, is small. The use of a removable appliance allows accurate plaque sampling from discrete enamel sites, whereas it is almost impossible to take uncontaminated samples from fissures and interproximal areas, the caries-prone sites in the natural dentition.

Furthermore, the use of enamel sections allows accurate quantification of changes in mineral content, as sites may be measured both pre- and post-experimentally.

Thus, although the problems outlined above have not been wholly overcome, the in situ appliance appears to be a suitable model for study of plaque microflora in relation to demineralisation, and further investigations, using a larger number of subjects, are indicated.

CHAPTER V.

COMPARISON OF CARIOGENIC POTENTIAL OF SIX STRAINS OF STREPTOCOCCUS MUTANS.

5.1. Introduction.

In recent years, much attention has been focused on attempting to identify accurately those patients most at risk of developing caries so that appropriate management may be undertaken to reduce this probability (reviewed by Bratthall & Carlsson, 1986; Blinkhorn & Geddes, 1987). Since caries is a multifactorial process, it is unlikely that a single test will be able to predict the likelihood of lesion development. At present, in addition to dietary assessment and recording previous caries experience, the other tests which are employed to help predict the susceptibility to dental caries include i] Strep. mutans and Lactobacillus spp. counts, ii] measurement of salivary flow rate, iii] assessment of salivary buffering capacity, and iv] determination of the sugar clearance rate from the oral cavity. Whilst many epidemiological studies have shown a positive correlation between the results of such tests and future caries activity on a group basis, their usefulness as predictors of future caries in individual patients remains in doubt (Blinkhorn & Geddes, 1987). Thus, while many human cross-sectional and longitudinal studies have shown a correlation between salivary and plaque counts of Strep. mutans and both caries prevalence and incidence (Zickert et al., 1982; Alaluusua & Renkonen, 1983; Lang et al., 1987), this is often not seen on an individual basis (Hardie et al., 1977; Loesche & Straffon, 1979; Russell, 1987).

The techniques employed in the in situ study described in Chapter IV allowed the proportion of different plaque bacteria, isolated from enamel sites, to be related to the degree of underlying demineralisation. Results showed that whilst overall, an association was evident between Strep. mutans levels and demineralisation, this relationship was not present with reference to individual sites. Many sites showed minimal changes in mineralisation although they were associated with high counts of Strep. mutans, while some sites developed mineral loss of over 500 Δz units despite Strep. mutans being undetected or comprising only a very small

proportion of the plaque flora. These findings were similar to those described in a number of longitudinal studies (Hardie et al., 1977; Masuda et al., 1979; Loesche et al., 1984).

The aim of this in vitro study was to determine whether variation existed in the cariogenic potential of strains of Strep. mutans isolated from six of the individuals involved in the above demineralisation study, and to ascertain whether this could help explain the lack of correlation between Strep. mutans salivary counts and caries experience, and between the plaque levels of this organism and the degree of underlying enamel demineralisation. A further aim was to determine whether the model showed a correlation between the cariogenic potential of the Strep. mutans strains and the caries activity of individuals from whom they had been isolated, and thus whether the model could have potential for development as an additional screening test to aid in the detection of high caries-risk individuals.

5.2. Study Design.

A modification of the model, described by Primrose and co-workers (1988) for assessing the cariogenicity of food was used, and strains of Strep. mutans isolated from six of the subjects involved in the in situ study were tested. The Strep. mutans type strain (NCTC 10449) used in the above food cariogenicity model was also tested for comparative purposes.

The strains were incubated with bovine enamel slabs and either a 5 % w/v sucrose solution or a 5 % w/v sorbitol control. An assessment of the acidogenic potential was determined by i] qualitative and quantitative measurement by isotachophoresis of the acids produced during the study, and ii] measurement of the pH of the system during the test period, whilst the demineralising potential was determined by i] measurement of the change in calcium concentration of the medium, and ii] radiographic and microdensitometric analysis of the enamel.

The results which were obtained from each strain were related to the subject's DMFS score and in situ demineralisation levels, and this relationship was then compared with the association found between the

initial screening test results described in Section 4.2.2. and the demineralisation produced using the appliance model.

5.3. Preparation of Bovine Enamel Slabs.

The bovine teeth which were used in each experimental run were obtained from a single animal. The lower incisors were removed from the mandible using a dental drill and diamond disc (2.5 cm diameter) and the teeth were then cleaned with pumice and alcohol as described in Section 2.3.1. Longitudinal slabs were cut, again using the dental drill and diamond disc, and the surface of the enamel was removed to a depth of approximately 100 μm by handgrinding as described in 4.4.2. The slabs were then coated with two layers of a proprietary nail varnish (Max Factor, London, England), leaving an exposed enamel window with dimensions of approximately 2 x 2 mm (Fig. 5.1.).

5.4. Preparation of Streptococcus mutans.

Strep. mutans cells had been isolated from each subject's mouth and freeze-dried as described in Section 2.6. In addition to these six strains, a freeze-dried Strep. mutans type culture (NCTC 10449) was used in this study. The ampoules were opened, the cells reconstituted in 2 ml ABB and inoculated on to blood agar plates, and the identity of each Strep. mutans strain checked using the API 20 Strep system as described in 2.5.5. Each strain was subcultured on to eight blood agar plates and incubated in 5 % CO_2 in air for 24 hours. The colonies were removed from the plates using sterile swabs with, in each case, the colonies from two plates being transferred to one of four plastic universal containers (Fig. 5.2.). Each universal contained 20 ml of a chemically defined growth medium with 5 % w/v glucose (Terlekyi, Willett and Shockman, 1975), (Appendix II). Chemicals for the medium were supplied by BDH Chemicals Ltd., Poole, Dorset, England. The cultures were then incubated overnight at 37°C in an orbital incubator at 100 rpm (Gallenkamp, Crawley, Sussex, England). After approximately 18 hours, the four universal bottles were centrifuged at 3000 rpm for 10 min in an MSE Centrifuge (Crawley, England) and the bacteria transferred to two universal containers, two pellets per container. These pellets were then washed with 20 ml 135 mM KCl, centrifuged as

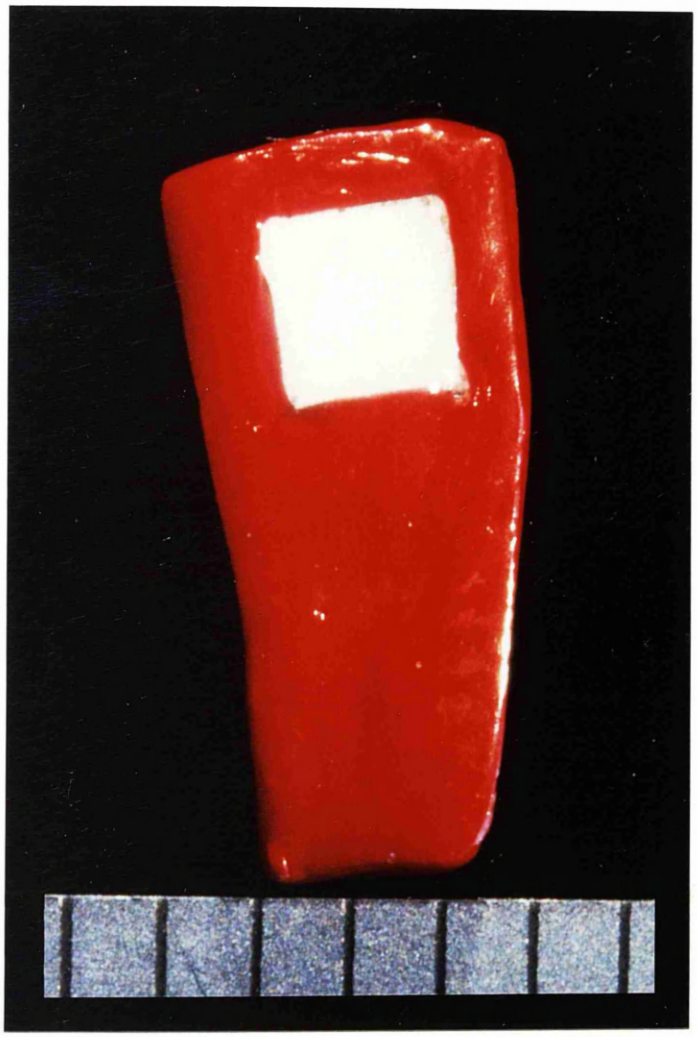


Fig. 5.1. **Bovine enamel slab, coated with varnish, leaving an exposed enamel window of approximately 2 x 2 mm.**

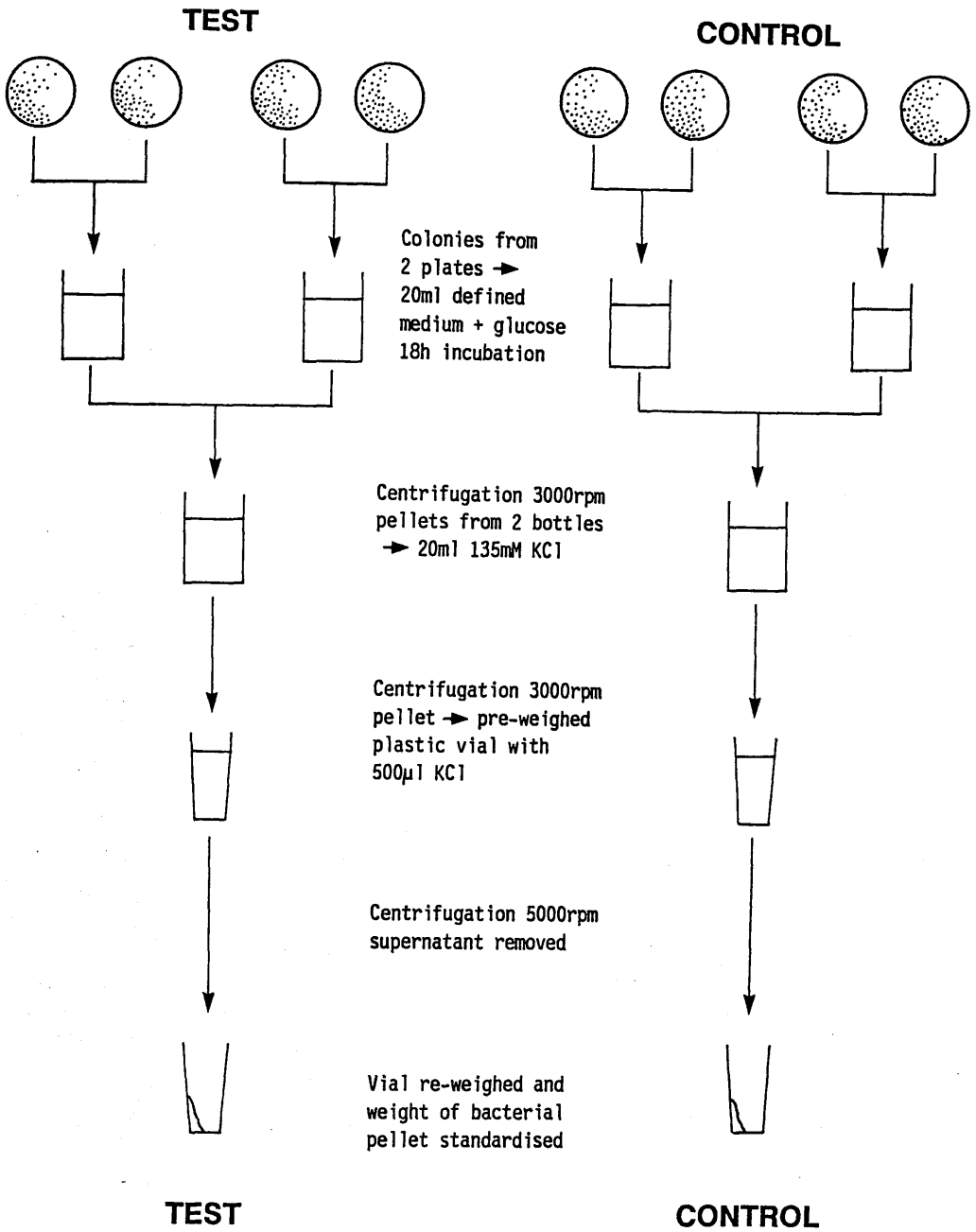


Fig. 5.2. **Diagramatic representation of preparation of Strep. mutans cells for in vitro testing of their cariogenic potential.**

above, and the cells then transferred to pre-weighed 1.5 ml capped polypropylene tubes and vortex-mixed with a further 500 µl of the KCl. The tubes were then centrifuged at 5000 rpm (MSE High Speed 18 Centrifuge, MSE Ltd., Crawley, England) for 10 min. The supernatant was removed and the vial re-weighed, and the weight of bacteria present in each vial standardised to allow valid comparisons to be made between the strains, in the experiments.

The prepared bovine enamel slabs and substrate were then added to the tubes (vide infra) and the experiment was run over a two day period, with the above procedure for the preparation of the cells being repeated on Day 2.

5.5. Experimental Protocols.

The methods used are shown in Fig. 5.3. The duration of each run was 2 days and, for each strain, 2 vials were prepared - one test and one sorbitol control.

After the weight of bacteria had been standardised, a bovine enamel slab was placed in each vial and the substrate, either a 5 % w/v sucrose solution or a 5 % w/v sorbitol solution, was added at a concentration of 3 µl/mg wet weight of bacteria. The pH of the vial contents was then recorded using a Corning pH meter and a 30 µl sample removed and placed in a labelled plastic tube which was then stored at a temperature of -20°C for subsequent acid anion analysis. In addition, a 10 µl sample was removed and placed in a plastic bijou containing 1 ml ABB. From this sample, the viable count of Strep. mutans cells present in each vial was determined (vide infra). The vial was then incubated in the orbital shaker at 37°C, and further pH readings and 30 µm samples, for isotachophoresis, were taken at 10 min, 30 min, 1, 5 and 24 hours. During this 24 hour period, bacteria were again prepared as described in Section 5.4. After 24 hours the enamel slab was removed from the vial, washed with sterile deionised water, gently dried and transferred to the fresh pellet of Strep. mutans cells. The substrate was then added, and the pH readings and samples taken, as described above.

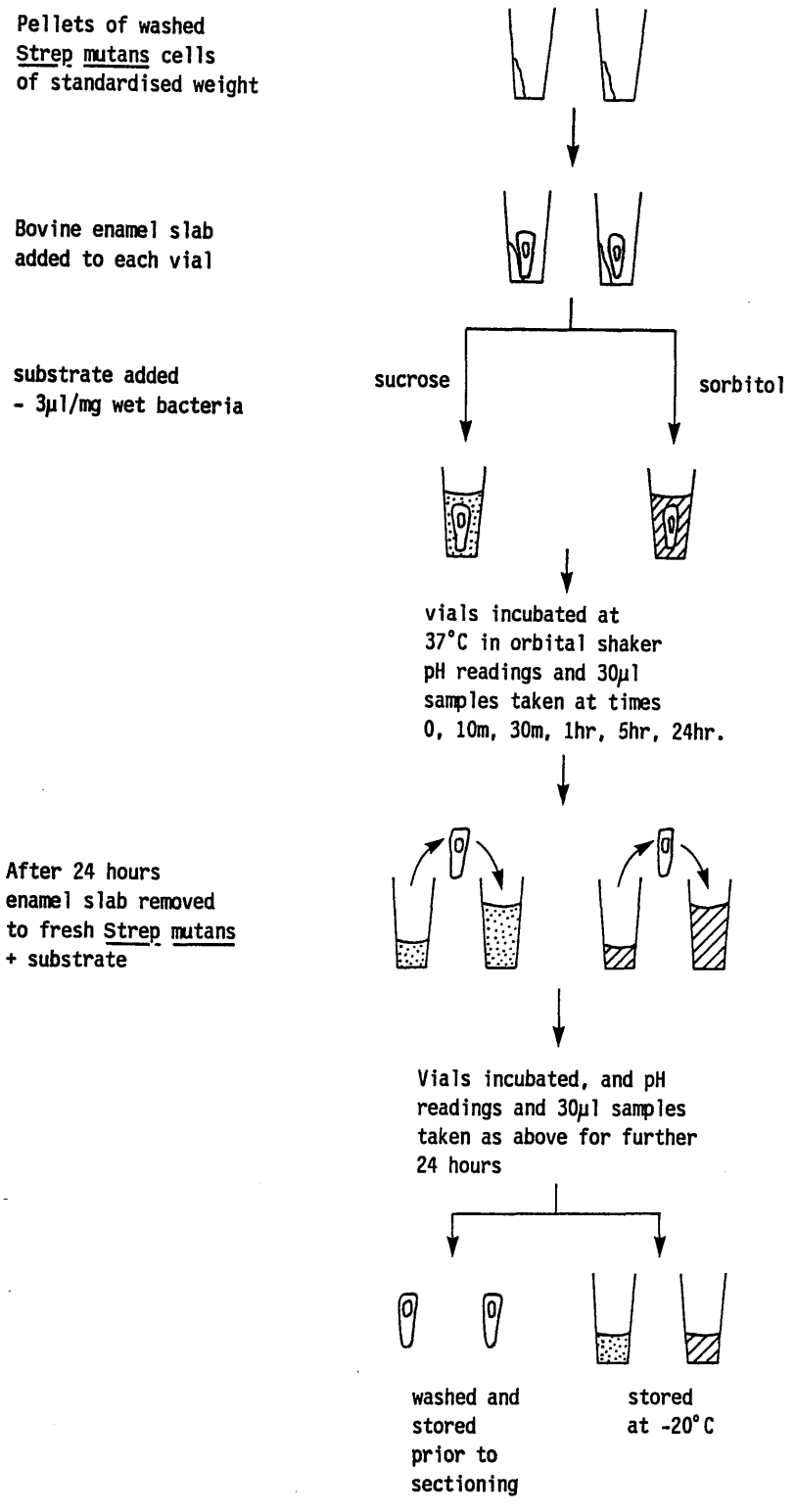


Fig. 5.3. **Diagramatic representation of incubation of bovine enamel slabs with substrate and Strep. mutans strain.**

After 48 hours, the enamel slab was removed from the vial and gently washed in deionised water. The vial containing the remainder of the slurry of cells and substrate was stored at -20°C.

5.5.1. Viable Count Determination.

The number of bacteria added to each vial was calculated by preparing in ABB serial ten-fold dilutions (10^{-2} - 10^{-7}) of the 10 µl sample, described in Section 5.5., with 50 µl of the 10^{-6} and 10^{-7} dilutions being spiral plated on to blood agar plates, as described in Section 2.5.4. After three day's incubation in 5 % CO_2 in air, the number of Strep. mutans colonies present on the plates was counted, and the viable count of Strep. mutans present in each vial determined.

5.6. Acid Anion Analysis.

The 30 µl and 24 hour samples which had been collected during the experiments were removed from the freezer and centrifuged at 5,000 rpm for 15 min. The supernatant fluid from each was then removed and placed in an individual plastic vial and stored at -20°C. The acids present in the samples were analysed by isotachophoresis using an LKB 2127 Tachophor (LKB Instruments, Broma, Sweden) as described previously in Section 2.7.1. Conversion equations were obtained by running standard acid solutions through the system, and these were used to convert the zone lengths produced by the different acids on the chart tracings into nmol concentrations of acid per mg wet weight of bacteria.

5.7. Calcium Assay.

Analysis of the calcium concentration present in the supernatant fluid obtained from the 0 and 24 hour samples of each test was also carried out. The Sigma Diagnostics Calcium Assay was used (Sigma Chemical Co Ltd., Poole, England). The method involved adding 10 µl of the sample to a chromogenic agent (o-cresolphthalein complexone) which would react with any calcium present to produce a purple colour, the intensity of the colour being proportional to the calcium concentration.

One microlitre volumes of the calcium assay solution were dispensed into microcuvets and 10 μl of the test sample was then added. The contents were mixed and the cuvet immediately placed in a Pye Unicam SP8-100 VV/VIS Spectrophotometer (Pye-Unicam Ltd., Cambridge, England) and the absorbance read at 575 nm. This value was then converted into a calcium concentration using an equation based on absorbance values obtained from standard calcium solutions.

5.8. Measurement of Mineral Content of Enamel Slabs.

At the end of the experiment, each slab was mounted on to an acrylic block and sectioned using a Leitz saw microtome as described in Section 2.3.2. The sections were then ground to a final thickeness of approximately 120 μm and microradiographed as described in Sections 2.3.3 and 2.4.1. The mineral content of the enamel was then assessed by microdensitometry, see Section 2.4.2., and the change estimated by subtracting the Δz value of an area of enamel under the varnish from the Δz value of the exposed enamel site.

5.9. Results

5.9.1. Viable Counts of Strep. mutans at Baseline.

The baseline viable counts (expressed as $\log_{10}$) of the Strep. mutans strains from the six subjects and the type culture are shown in Table 5.1. The mean initial counts from the experimental run on each of four days, for all the strains in both sucrose and sorbitol groups showed less than a ten-fold increase from lowest to highest value, with the counts in sucrose samples ranging from 9.39 to 9.98, and those in sorbitol from 9.55 to 9.92.

5.9.2. Acid Anion Concentrations Under Sucrose Conditions.

The mean acid anion concentrations present over the 24 hour experimental periods for each strain, under sucrose conditions, are shown in Table 5.2. Each strain showed a large rise in total acid from baseline to 24 hours, with the values at baseline ranging from 18.5 nmol/mg wet weight bacteria (strain B) to 48.0 (strain D), and the range at 24 hours being from 105.4 (strain B) to 303.6 (strain E). Lactate comprised the majority of the

identifiable acid, from each strain, at each sampling time, with the mean percentage of the total acid, for all strains combined, ranging from 62 % at baseline to 80 % at 24 hours (see Table 5.3.). The levels of acetate showed the opposite trend, with a decrease in mean percentage of the total acid from 25 % at baseline, to 12 % at 24 hours.

These two acids comprised a very large proportion of the total acid, while the concentrations of formate and propionate were very small, not exceeding 1.0 nmol/mg wet weight bacteria at any sampling time (see Table 5.2.). The concentrations of pyruvate and succinate both rose with time with each strain of Strep. mutans, but their proportions relative to the total acid concentration fell by up to 50 % over the 24 hour periods.

The mean total acid concentration at 24 hours for strains isolated from Subjects A - G are shown in Fig. 5.4. Large differences amongst the strains were found, with the values fitting into three groups. The lowest category comprised strains A, B and G, with the Strep. mutans strain from Subject B producing least acid (105 nmol/mg wet weight bacteria). The second group contained strains D and F, which produced similar mean concentrations of total acid (228 and 236 nmol/mg wet weight, respectively), while the highest total acid concentration was produced by Subject E. Student's t-test analysis showed that this strain's mean acid concentration of 304 nmol/mg wet weight bacteria, was significantly higher than each of the other strains, and that each of the strains in the other groups was significantly different from the Strep. mutans strains in the other two groups (see Fig. 5.4.).

5.9.3. pH Measurements Over 24 Hours For Each Strep. mutans Strain Under Sucrose Conditions.

Table 5.4. shows the pH measurements recorded for each strain during the four 24 hour periods of incubation with 5 % sucrose. The initial mean pH readings ranged from 3.78 with strain E, to 4.48 for the strain isolated from Subject A. Fig. 5.5. shows the mean pH values for each strain over the 24 hour period, with Table 5.5. showing the means of the minimum and final pH values for each strain. The minimum pH values fitted into the same groups as was found with total acid concentration, with Subject E having the lowest pH minimum, of 3.38, D and F having intermediate

values of pH 3.48 and 3.52 respectively, while the minimum pH measurements of strains A, B and G ranged from 3.63 to 3.66. The minimum pH occurred at 30 min for strains D, E and F, and at 1 hour for the other three strains.

The lowest 24 hour pH value was also obtained from the Strep. mutans strain isolated from Subject E (3.82), the highest value of 4.32 was associated with strain G, whilst the measurements obtained from the other strains were similar, ranging from 4.10 to 4.18.

5.9.4. Mineral Loss Associated With Strep. mutans Strains During Sucrose Incubation.

The change in calcium concentration of the slurry of bacteria and sucrose, over 24 hour periods, for each strain, is shown in Table 5.6. The values obtained from the first 24 hours of each two day experiment are shown in columns 1 and 3, with the 24 to 48 hour measurements being found in columns 2 and 4. As the mean values obtained from these two groups were very similar, 8.66 mM (0 - 24 hours) and 8.54 mM (24 - 48 hours), they have been combined into the one group for each strain, and the mean calculated.

These values, of mean change in calcium concentration over the 24 hour periods, is shown in Fig. 5.6. The results showed a similar pattern to total acid concentration and minimum pH values, with the Strep. mutans strain from Subject E being associated with the highest calcium release from the bovine enamel, and strains A and B showing the smallest change in calcium concentration. Student's t-test analysis showed significant differences between strain E and each strain in the A, B and G group, and strains A and B were also significantly lower than D and F, and D respectively.

The Δz values obtained from the bovine enamel are shown in Table 5.7. These values represent the change in mineral content over each 48 hour experiment. Attempts were made to obtain three enamel sections from each slab, but this was not always possible, hence six sections were not always available for each Strep. mutans strain analysis.

The mean Δz values associated with each Subject's strain is shown in Fig. 5.7. The strains belonging to the A, B and G group again showed the smallest change in mineral content, with in this case, strain G having the lowest Δz score, and strain A, the highest of the three, having a large standard deviation. While the mean Δz value of strain E was slightly higher than that associated with strain D, it was lower than that of the Strep. mutans isolated from Subject F, the values being 2121 and 2781 % vol min x μm, respectively. Although the mean Δz values showed marked differences between some strains, the large standard deviations resulted in none of the differences reaching significant levels (Student's t-test).

5.9.5. Acid Anion Concentrations Under Sorbitol Conditions.

The lactate, acetate and total acid concentrations produced by the different strains of Strep. mutans during incubation with 5 % sorbitol are shown in Table 5.8. The concentrations of lactate and total acid produced by the bacteria were significantly lower with sorbitol substrate than the corresponding values at each sampling time, for each subject's strain during incubation with sucrose. The total acid concentration at 24 hours under sorbitol conditions, as a proportion of the total acid produced by the Strep. mutans in the presence of sucrose, ranged from 33 % in strain B, to 48 % in strain E. The corresponding values for lactate ranged from 14 % (Subject G) to 33 % in Subject E.

However, in five of the six strains, the concentration of acetate was higher following 24 hour incubation with sorbitol, the greatest increase being seen with strain D (149 %), while in strain B, the acetate concentration under sorbitol conditions was only 55 % of the concentration obtained following incubation with sucrose.

The proportions of lactate and acetate, expressed as percentages of the total acid, under sorbitol conditions, are shown in Table 5.9. The mean proportion of lactate, for all strains combined, varied little with time, being on each occasion approximately 50 %, compared to the increase in proportion with time (62 % to 80 %) seen with sucrose conditions. While the acetate proportion decreased from 25 % at baseline to 12 % at 24 hours with sucrose, an increase in acetate from 30 % to 40 % over the

first five hours, with a slight fall to 38 % at 24 hours, was seen during incubation with sorbitol.

While for each strain, the concentration of lactate and total acid were higher with sucrose incubation than with sorbitol, the lactate from strain E, under the latter, was greater than the lactate concentration produced by strain B with sucrose. Likewise, strain E's total acid with sorbitol was considerably greater than the total acid produced by strain B, and similar to that produced by strains A and G, under sucrose conditions.

Comparison of total acids produced by the six strains following 24 hour sorbitol incubation showed that, as with sucrose, differences were seen between the strains, with strain E producing the greatest amount of acid (144 nmol/mg wet weight bacteria), F and D producing a similar but lesser amount (92 and 86, respectively), A and G less again (52 and 49), and B producing the least amount of acid (35 nmol/mg wet weight bacteria).

5.9.6. pH Measurement Over 24 Hours for Each Strep. mutans Strain Under Sorbitol Conditions.

For each strain of Strep. mutans, the pH measurements over 24 hours, during sorbitol incubation, are shown in Table 5.10. The lowest baseline pH was seen with strain E (3.72), with strain F's pH slightly higher at 3.84. The highest baseline pH was seen, as before, with strain A (4.44). For the strains A and B, the pH fell slightly in the first 10 and 30 min respectively following the addition of the substrate while, with the strains from all other subjects, the pH rose from the baseline value, the maximum rise occurring with strains E and F (ΔpH 0.72 and 0.75 respectively).

5.9.7. Mineral Loss Associated With Strep. mutans Strains During Sorbitol Incubation.

The mean changes in calcium concentration of the slurry of Strep. mutans and sorbitol for each strain is shown in Table 5.11. The greatest amount of calcium release occurred with strain E (4.78 mM), while the mean change in calcium for other strains ranged from 0.98 mM (type culture) to 2.88 mM with strain D. The mean values obtained for the different

strains were all lower than those obtained with sucrose incubation (range 6.32 - 10.92 mM).

The greatest change in mineral content, as measured by Δz was 823 units (Subject E), the levels seen with strains A and F being similar (see Table 5.12.). The Δz values for strains B and D were approximately 300 units, and for strain G and type culture, less than 40 units. All these values are considerably lower than even the lowest Δz (viz. 1166 units), obtained with sucrose incubation.

5.9.8. Relationship Between *in situ* Demineralisation Levels and Cariogenicity Test Parameters.

A plot of the mean total acid anion concentration produced by each strain at 24 hours following incubation with sucrose, against the mean level of *in situ* demineralisation produced by the subject under normal and sucrose plaque conditions, (as described in Chapter IV), is shown in Fig. 5.8. A linear increase in total acid concentration with increasing Δz score was seen, with the correlation between the parameters being highly significant ($p < 0.001$ - linear regression analysis).

Shown in Fig. 5.9. is the relationship between the mean minimum pH produced by each *Strep. mutans* strain, and the subject's Δz score, where the correlation between these was again significant ($p < 0.01$).

The plots of the mean calcium release and Δz values obtained from bovine slabs following incubation with the six *Strep. mutans* strains and sucrose, against the *in situ* Δz scores from the human enamel sections, are shown in Figs. 5.10. and 5.11., respectively. Both showed that an increase in mineral loss from the bovine enamel in the *in vitro* cariogenicity model was associated with an increased Δz score in the *in situ* appliance model. While this correlation was significant at the 5 % level for calcium release, the relationship for Δz scores of the bovine enamel did not reach significant levels.

5.9.9. Relationship Between DMFS Scores of Individuals and Cariogenicity Test Parameters.

The values of the total acid concentration at 24 hours, minimum pH, calcium release and Δz values of the bovine enamel slabs, obtained from the in vitro cariogenicity test model, were also plotted against the DMFS scores of the individuals, as shown in Figs. 5.12. to 5.15. A linear relationship between these parameters was again seen, with significant correlation being found between DMFS scores and total acid concentration ($p < 0.01$), and minimum pH and calcium release (both $p < 0.05$), while the association with Δz scores of the bovine enamel did not reach significant levels ($0.1 > p > 0.05$).

A summary of the results of linear regression analysis between the DMFS and in situ demineralisation scores, and both the in vitro cariogenicity test parameters and the salivary characteristics of the individuals (detailed in Chapter IV), is shown in Table 5.13. While significant correlations were found between three of the cariogenicity test parameters and natural and experimental mineral loss experienced by the subjects, none of the associations between salivary characteristics and DMFS scores or in situ demineralisation reached significance at the 5 % level.

SUCROSE

	1	2	3	4	Mean (SD)
A	9.66	9.81	10.11	10.34	9.98 (0.30)
B	9.41	9.00	9.38	9.76	9.39 (0.31)
D	9.40	9.15	9.71	9.60	9.46 (0.25)
E	10.15	9.58	9.32	9.58	9.66 (0.35)
F	9.66	9.66	9.63	9.26	9.55 (0.20)
G	9.92	9.41	9.66	9.71	9.68 (0.21)
TC	9.69	10.00	9.66	9.73	9.77 (0.16)

SORBITOL

	1	2	3	4	Mean (SD)
A	9.34	9.96	10.00	10.36	9.92 (0.42)
B	9.83	9.56	9.23	9.58	9.55 (0.25)
D	9.58	9.48	9.76	9.48	9.58 (0.13)
E	9.89	9.58	9.40	9.69	9.64 (0.20)
F	9.50	10.04	9.30	9.92	9.69 (0.35)
G	9.95	9.66	9.92	10.18	9.93 (0.21)
TC	9.76	9.73	9.76	9.50	9.69 (0.12)

Table 5.1. Log_{10} viable counts of Strep. mutans added to vials for incubation with 5 % w/v sucrose and 5 % w/v sorbitol, on each of four occasions, for Subjects A - G and Type Culture (NCTC 10449).

Table 5.2. Mean (SD) acid anion concentration (nmol/mg wet weight bacteria) at all sampling times from 0 - 24 h, with 5 % w/v sucrose for Strep. mutans strains from Subjects A - G and Type Culture, n=4.

SUBJECT A
SUCROSE

	0	10m	30m	1h	5h	24h
Formate	0.11 (0.22)	0.12 (0.24)	0.20 (0.41)	0.36 (0.73)	0.43 (0.86)	0.91 (1.82)
Pyruvate	2.55 (0.31)	2.30 (0.86)	2.62 (0.74)	4.19 (1.04)	7.14 (0.89)	11.38 (2.39)
Phosphate	3.68 (2.73)	2.38 (1.05)	3.36 (1.22)	5.52 (1.71)	8.72 (1.05)	22.79 (4.20)
Lactate	10.72 (5.40)	21.30 (11.05)	39.44 (13.37)	60.15 (15.85)	102.39 (20.03)	112.39 (5.08)
Succinate	0.78 (0.15)	0.82 (0.12)	1.08 (0.48)	1.74 (0.52)	2.20 (0.81)	4.14 (0.73)
Acetate	4.83 (1.64)	5.57 (2.02)	7.74 (2.60)	10.10 (2.72)	13.25 (3.72)	16.02 (2.62)
Propionate	0.02 (0.05)	0.18 (0.15)	0.11 (0.10)	0.18 (0.26)	0.19 (0.20)	0.21 (0.34)
Total acid	19.01 (7.03)	30.29 (12.30)	49.97 (17.64)	76.73 (19.17)	125.60 (24.89)	148.25 (10.13)

SUBJECT B
SUCROSE

	0	10m	30m	1h	5h	24h
Formate	0.18 (0.30)	0.10 (0.17)	0.00	0.00	0.00	0.29 (0.50)
Pyruvate	1.55 (0.55)	1.44 (0.19)	1.70 (0.40)	2.87 (0.75)	5.31 (2.07)	6.04 (3.64)
Phosphate	2.97 (1.43)	3.64 (1.44)	3.60 (2.18)	5.05 (1.85)	9.26 (3.63)	20.02 (6.88)
Lactate	10.02 (4.08)	17.60 (4.71)	23.97 (5.39)	45.07 (21.30)	65.13 (27.22)	77.02 (23.94)
Succinate	0.61 (0.11)	0.45 (0.08)	0.72 (0.35)	0.92 (0.19)	1.72 (0.29)	2.98 (0.98)
Acetate	6.18 (2.83)	7.55 (3.87)	7.68 (3.14)	10.88 (6.28)	16.42 (10.64)	19.22 (10.79)
Propionate	0.00	0.02 (0.02)	0.05 (0.07)	0.04 (0.07)	0.11 (0.16)	0.00
Total acid	18.54 (7.00)	27.14 (8.51)	34.11 (8.18)	73.93 (14.07)	88.70 (39.16)	105.37 (32.66)

SUBJECT D
SUCROSE

	0	10m	30m	1h	5h	24h
Formate	0.45 (0.65)	0.26 (0.30)	0.00	0.12 (0.24)	0.00	0.50 (0.94)
Pyruvate	6.50 (1.71)	5.57 (1.39)	6.10 (1.76)	9.14 (4.77)	12.77 (3.45)	22.64 (14.54)
Phosphate	9.38 (4.23)	9.49 (5.64)	10.88 (5.55)	12.87 (7.36)	22.39 (4.83)	36.40 (5.15)
Lactate	30.37 (13.05)	35.20 (19.00)	72.74 (43.34)	102.44 (55.91)	144.54 (36.49)	188.98 (37.00)
Succinate	1.18 (0.48)	1.44 (0.70)	1.37 (1.01)	1.80 (0.60)	2.94 (1.47)	3.66 (2.35)
Acetate	9.36 (1.71)	10.50 (4.66)	13.21 (7.70)	17.51 (12.34)	18.05 (10.59)	18.60 (8.47)
Propionate	0.17 (0.27)	0.10 (0.18)	0.00	0.27 (0.50)	0.08 (0.16)	0.22 (0.44
Total acid	48.02 (14.12)	53.28 (23.41)	93.38 (50.16)	131.28 (71.39)	178.38 (48.78)	227.95 (48.45)

SUBJECT E
SUCROSE

	0	10m	30m	1h	5h	24h
Formate	0.26 (0.41)	0.43 (0.34)	0.00	0.00	0.00	0.00
Pyruvate	3.21 (1.63)	4.04 (1.71)	5.19 (0.47)	8.21 (3.18)	10.99 (1.42)	16.30 (1.74)
Phosphate	5.44 (1.84)	6.94 (2.29)	7.35 (0.45)	11.44 (0.44)	19.83 (2.50)	27.39 (3.87)
Lactate	34.42 (13.51)	48.22 (11.62)	80.87 (14.31)	122.38 (27.40)	192.54 (16.95)	253.90 (18.55)
Succinate	0.80 (0.38)	1.19 (0.52)	1.26 (0.93)	2.35 (1.50)	2.56 (1.35)	2.88 (1.15)
Acetate	8.40 (2.44)	11.48 (1.27)	13.88 (0.59)	18.62 (2.91)	25.24 (5.98)	30.56 (9.13)
Propionate	0.02 (0.03)	0.04 (0.09)	0.00	0.18 (0.35)	0.22 (0.44)	0.00
Total acid	47.10 (16.42)	65.40 (13.72)	101.21 (13.04)	151.73 (30.04)	231.56 (21.76)	303.64 (26.35)

Table 5.2. cont.

SUBJECT F
SUCROSE

	0	10m	30m	1h	5h	24h
Formate	0.52 (0.62)	0.57 (0.85)	0.45 (0.90)	0.45 (0.90)	0.26 (0.52)	0.56 (0.75)
Pyruvate	2.81 (1.22)	3.46 (0.77)	4.58 (0.17)	6.04 (1.82)	10.78 (3.07)	13.91 (4.41)
Phosphate	4.04 (1.88)	5.03 (1.87)	6.02 (1.42)	8.48 (3.20)	17.78 (3.50)	30.47 (3.16)
Lactate	27.32 (6.46)	36.08 (7.66)	51.69 (9.70)	79.90 (21.29)	134.40 (28.02)	188.16 (26.29)
Succinate	0.94 (0.53)	1.25 (0.85)	1.32 (0.90)	1.63 (0.48)	2.56 (1.47)	3.23 (1.84)
Acetate	8.26 (2.04)	9.80 (1.46)	14.09 (3.38)	19.65 (6.67)	24.34 (6.57)	29.69 (6.74)
Propionate	0.06 (0.08)	0.29 (0.49)	0.00	0.10 (0.17)	0.00	0.03 (0.06)
Total acid	39.90 (7.28)	51.45 (7.78	72.14 (12.48)	107.78 (28.82)	172.35 (31.51)	235.57 (31.41)

SUBJECT G
SUCROSE

	0	10m	30m	1h	5h	24h
Formate	0.50 (0.19)	0.52 (0.44)	0.24 (0.48)	0.64 (0.68)	0.34 (0.68)	0.93 (1.14)
Pyruvate	2.10 (0.78)	1.96 (0.60)	5.42 (6.44)	4.99 (2.06)	4.32 (1.68)	8.61 (4.78)
Phosphate	4.46 (1.74)	3.96 (2.44)	6.29 (1.91)	7.51 (1.88)	15.11 (3.84)	28.06 (10.03)
Lactate	18.32 (10.29)	23.80 (10.24)	47.56 (19.28)	67.34 (27.49)	86.32 (17.28)	110.52 (25.69)
Succinate	0.55 (0.23)	0.76 (0.33)	0.94 (0.42)	1.44 (0.37)	1.75 (0.42)	3.08 (1.35)
Acetate	9.08 (1.90)	9.06 (2.36)	12.30 (3.83)	14.89 (4.95)	16.74 (4.47)	19.68 (4.14)
Propionate	0.04 (0.06)	0.12 (0.23)	0.15 (0.23	0.27 (0.29)	0.04 (0.06)	0.22 (0.44)
Total acid	30.56 (11.85)	36.24 (11.79)	66.61 (19.19)	89.56 (33.66)	109.52 (21.91)	143.05 (32.52)

Table 5.2. cont.

TYPE CULTURE
SUCROSE

	0	10m	30m	1h	5h	24h
Formate	0.17 (0.21)	0.08 (0.11)	0.16 (0.23)	0.00	0.00	0.84 (0.78)
Pyruvate	1.70 (0.22)	1.97 (0.18)	2.59 (0.72)	3.09 (0.30)	2.98 (1.04)	4,23 (1.38)
Phosphate	3.46 (0.97)	2.46 (0.83)	2.68 (0.25)	3.40 (0.32)	12.50 (3.80)	15.21 (2.49)
Lactate	13.60 (2.21)	16.73 (1.23)	27.10 (5.77)	42.42 (9.09)	74.19 (0.35)	115.18 (0.22)
Succinate	0.32 (0.05)	0.34 (0.06)	0.35 (0.13)	0.54 (0.00)	0.88 (0.33)	1.28 (0.40)
Acetate	6.72 (1.20)	6.64 (1.33)	6.36 (0.98)	8.44 (0.81)	10.00 (2.01)	15.88 (4.24)
Propionate	0.00	0.00	0.00	0.00	0.00	0.00
Total acid	22.52 (3.89)	25.76 (2.54)	36.56 (7.83)	54.49 (10.20)	88.05 (3.03)	137.40 (5.03)

Table 5.2. cont.

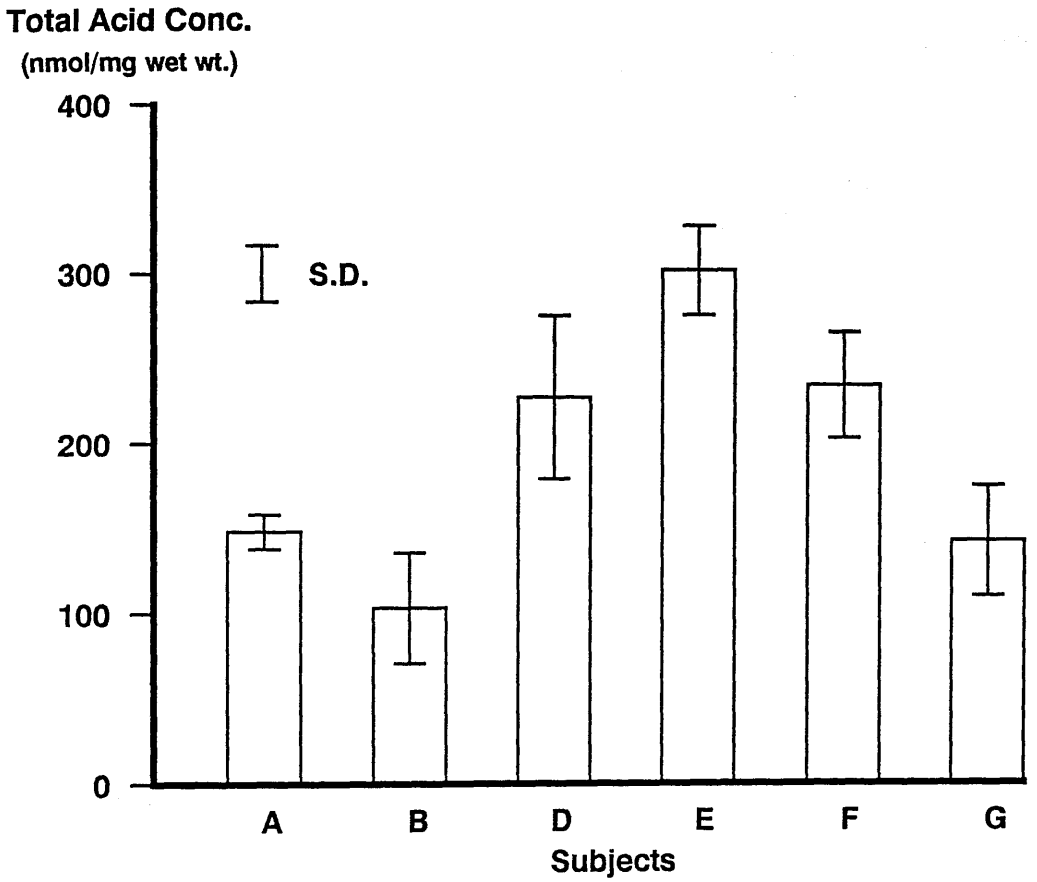
LACTATE

	0	10m	30m	1h	5h	24h
A	56	70	79	78	82	76
B	54	65	70	61	73	73
D	63	66	78	78	81	83
E	73	74	80	81	83	84
F	68	70	72	74	78	80
G	60	66	71	75	79	77
TC	60	65	74	78	84	84
Mean	62	68	75	75	80	80

ACETATE

	0	10m	30m	1h	5h	24h
A	25	18	16	13	10	11
B	33	28	22	15	18	18
D	20	20	14	13	10	8
E	18	18	14	12	11	10
F	21	19	20	18	14	13
G	30	25	18	17	15	14
TC	30	26	17	16	11	12
Mean	25	22	17	15	13	12

Table 5.3. Proportion of lactate and acetate, expressed as a percentage of the total acid, at each sampling time from 0 - 24 h, under sucrose conditions, for Strep. mutans strains from Subjects A - G and Type Culture.



E - v - ***A ; - ***B ; - *D ; - *F ; - ***G

D - v - *A ; - **B ; - *G

F - v - **A ; - **B ; - **G

* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

Fig. 5.4. **Mean (SD) total acid anion concentration (nmol/mg wet weight bacteria) at 24 h, for Strep. mutans strains isolated from Subjects A - G, n=4.**

Table 5.4. pH measurements at each sampling time from 0 - 24 h, during incubation with 5 % w/v sucrose, for Strep. mutans strains from Subjects A - G and Type Culture, n=4.

SUBJECT A
SUCROSE

	0	10m	30m	1h	5h	24h
1	4.28	3.90	3.71	3.67	3.77	4.18
2	4.56	3.93	3.76	3.72	3.68	4.18
3	4.72	4.04	3.74	3.67	3.75	4.06
4	4.37	3.97	3.68	3.59	3.62	3.98
Mean (SD)	4.48 (0.20)	3.96 (0.10)	3.72 (0.04)	3.66 (0.05)	3.70 (0.10)	4.10 (0.10)

SUBJECT B
SUCROSE

	0	10m	30m	1h	5h	24h
1	4.21	3.96	3.81	3.67	3.70	4.13
2	4.28	3.94	3.79	3.74	3.73	4.05
3	4.19	3.74	3.56	3.59	3.69	4.13
4	4.03	3.69	3.55	3.55	3.82	4.40
Mean (SD)	4.18 (0.10)	3.83 (0.10)	3.68 (0.10)	3.64 (0.10)	3.74 (0.05)	4.18 (0.20)

SUBJECT D
SUCROSE

	0	10m	30m	1h	5h	24h
1	4.08	3.80	3.54	3.70	3.63	4.00
2	4.15	3.76	3.74	3.47	3.67	4.01
3	4.28	3.74	3.37	3.33	3.69	4.25
4	3.96	3.28	3.28	3.47	3.87	4.15
Mean (SD)	4.12 (0.10)	3.64 (0.20)	3.48 (0.20)	3.49 (0.15)	3.72 (0.10)	4.10 (0.10)

SUBJECT E
SUCROSE

	0	10m	30m	1h	5h	24h
1	3.85	3.78	3.55	3.51	3.66	3.98
2	4.16	3.99	3.54	3.52	3.64	3.83
3	3.48	3.17	3.13	3.31	3.66	3.83
4	3.61	3.31	3.29	3.33	3.51	3.66
Mean (SD)	3.78 (0.30)	3.56 (0.39)	3.38 (0.20)	3.42 (0.10)	3.62 (0.10)	3.82 (0.10)

SUBJECT F
SUCROSE

	0	10m	30m	1h	5h	24h
1	4.05	3.74	3.63	3.56	3.73	4.15
2	4.14	3.78	3.64	3.61	3.63	3.96
3	3.95	3.44	3.36	3.42	3.71	4.07
4	3.48	3.30	3.45	3.57	3.84	4.38
Mean (SD)	3.90 (0.30)	3.56 (0.20)	3.52 (0.10)	3.54 (0.10)	3.73 (0.10)	4.14 (0.20)

SUBJECT G
SUCROSE

	0	10m	30m	1h	5h	24h
1	4.36	4.14	3.73	3.64	3.78	4.34
2	4.56	4.25	3.83	3.67	3.83	4.34
3	3.82	3.61	3.55	3.61	4.01	4.56
4	3.60	3.68	3.63	3.61	3.71	4.06
Mean (SD)	4.08 (0.40)	3.92 (0.30)	3.70 (0.10)	3.63 (0.02)	3.83 (0.10)	4.32 (0.20)

Table 5.4. cont.

TYPE CULTURE
SUCROSE

	0	10m	30m	1h	5h	24h
1	4.19	3.65	3.35	3.52	3.50	4.07
2	3.90	3.81	3.53	3.40	3.42	3.92
3	4.03	3.79	3.58	3.57	3.63	4.22
4	4.11	3.78	3.67	3.58	3.63	4.39
Mean (SD)	4.06 (0.10)	3.76 (0.10)	3.54 (0.10)	3.52 (0.10)	3.54 (0.10)	4.15 (0.20)

Table 5.4. cont.

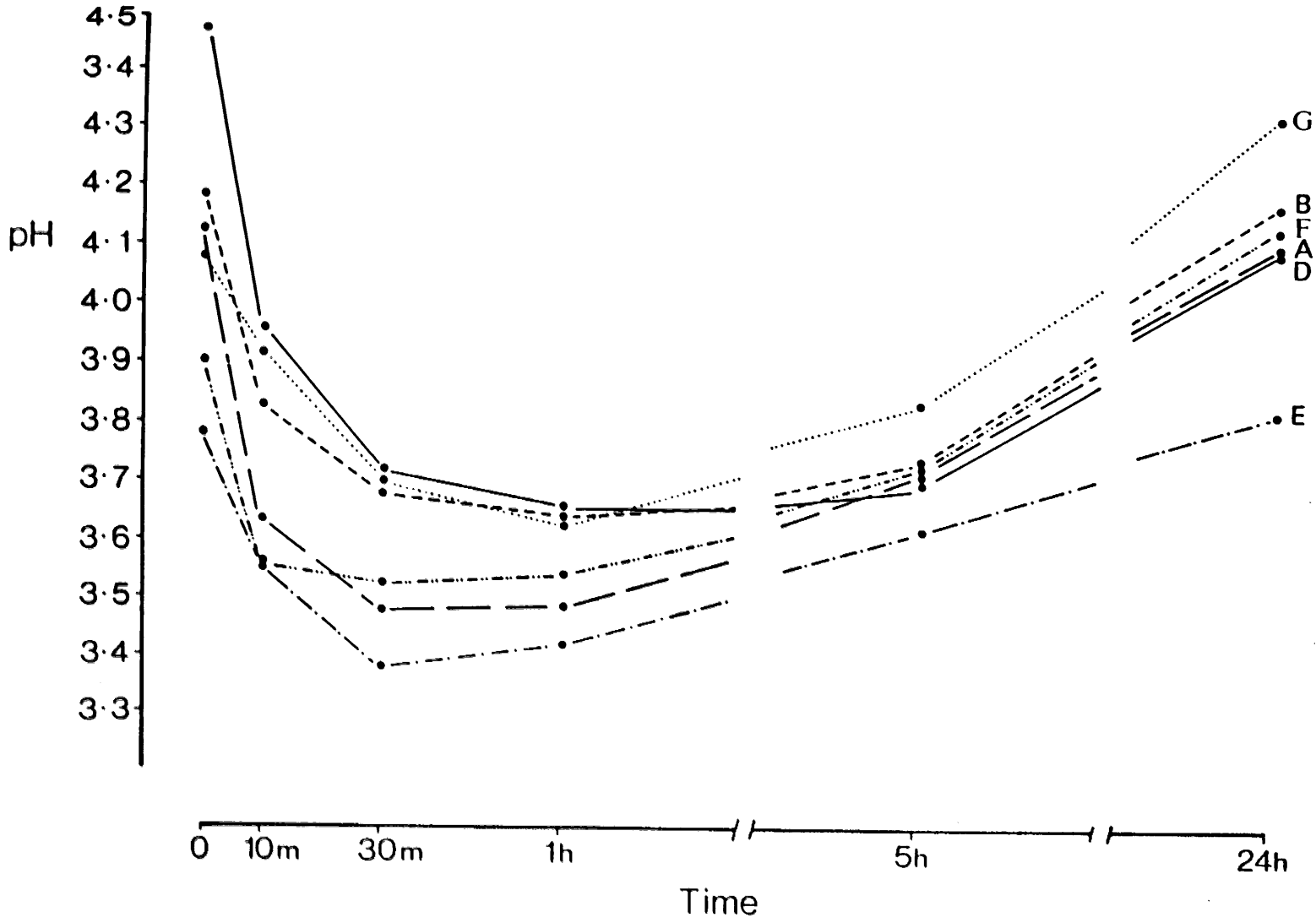


Fig. 5.5. **Mean pH measurements at each sampling time from 0 - 24 h during incubation with 5 % w/v sucrose, for Strep. mutans strains from Subjects A - G, n=4.**

	Minimum pH Mean (SD)	Final pH Mean (SD)
A	3.66 (0.05)	4.10 (0.10)
B	3.64 (0.08)	4.18 (0.15)
D	3.48 (0.20)	4.10 (0.12)
E	3.38 (0.13)	3.82 (0.13)
F	3.52 (0.10)	4.14 (0.20)
G	3.63 (0.02)	4.32 (0.22)
TC	3.52 (0.08)	4.15 (0.21)

Table 5.5. Mean minimum and final pH values obtained from Strep. mutans strains from Subjects A - G and Type Culture, during incubation with 5 % w/v sucrose, n=4.

CALCIUM RELEASE
SUCROSE

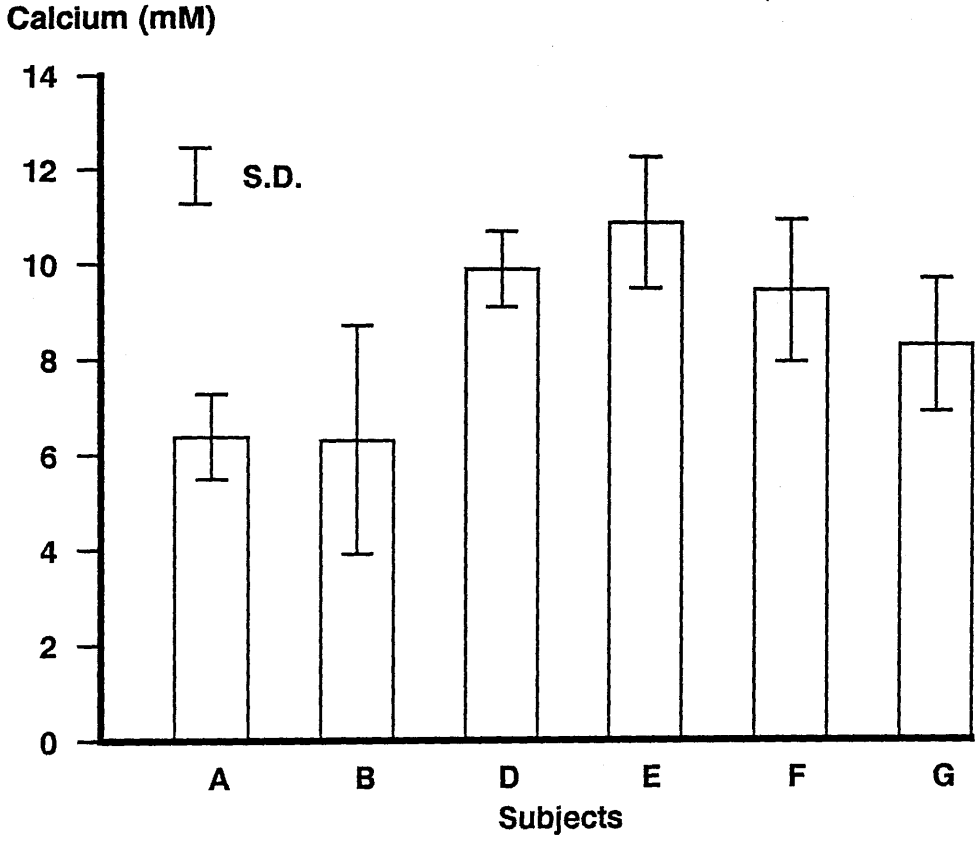
	1	2	3	4	Mean (SD)
A	6.76	5.56	5.78	7.46	6.39 (0.9)
B	4.73	3.86	8.86	7.82	6.32 (2.4)
D	11.07	9.34	9.48	9.83	9.93 (0.8)
E	11.64	9.07	10.73	12.04	10.92 (1.4)
F	8.82	7.94	9.69	11.56	9.50 (1.5)
G	9.79	6.52	8.45	8.67	8.36 (1.4)
TC	6.87	10.83	8.64	9.06	8.85 (1.6)

Table 5.6. Change in calcium concentration (mM) of slurry over 24 h periods following incubation of Strep. mutans, 5 % w/v sucrose and bovine tooth slab, for Subjects A - G and Type Culture, n=4.

Δz SCORE
SUCROSE

	1	2	3	4	5	6	Mean (SD)
A	2308	2454	1103	1323	1486	1585	1792 (680)
B	1319	1687	544	1687	1851	1315	1400 (470)
D	2093	2682	2025	1929	1577	-	2061 (400)
E	2548	2283	2369	1918	1241	2365	2121 (480)
F	3636	3762	2813	2189	2000	2286	2781 (760)
G	1095	1796	1439	694	1371	6011	166 (460)
TC	1935	2164	1756	1953	-	-	1952 (167)

Table 5.7. Δz (% vol min x μm) of enamel sections obtained from bovine slabs incubated for 48 h with Strep. mutans and 5 % w/v sucrose, for Subjects A - G and Type Culture, n=6.



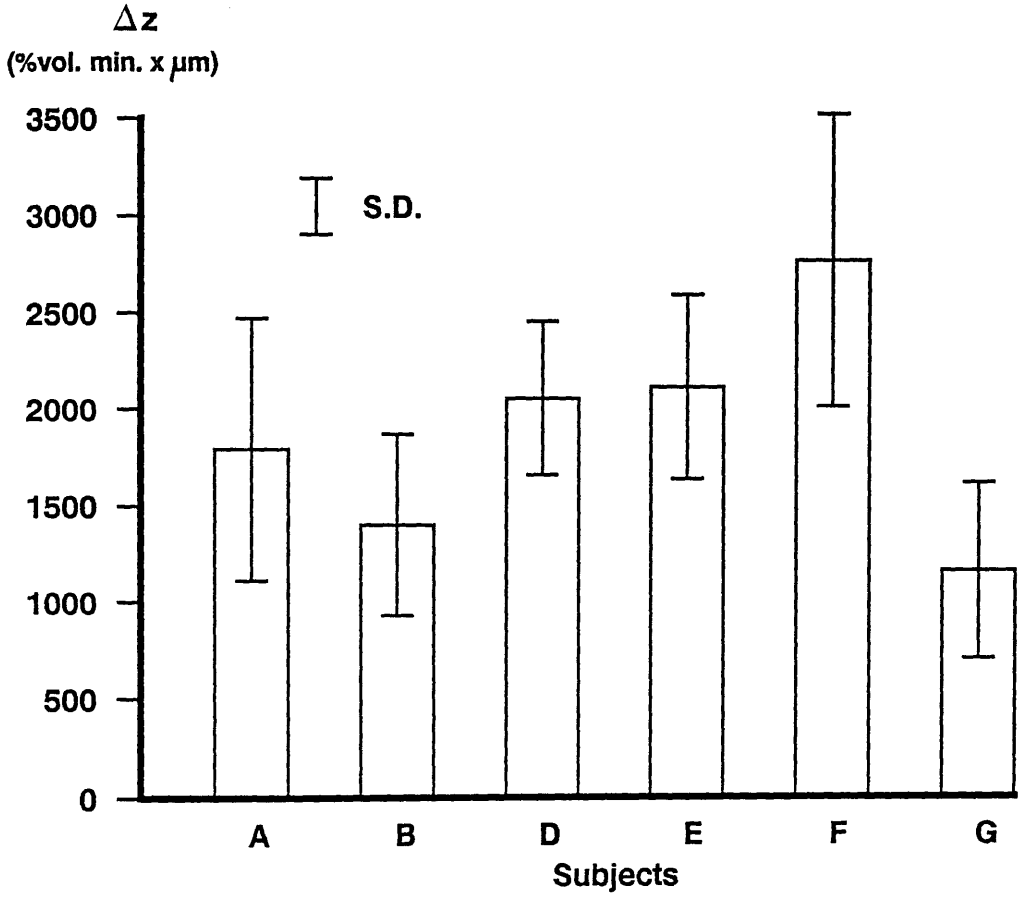
E - v - A** ; - B* ; - G*

D - v - A** ; - B*

F - v - A*

* = $p < 0.05$; ** = $p < 0.01$

Fig. 5.6. **Mean (SD) change in calcium concentration (mM) of slurry of Strep. mutans and sucrose, incubated with bovine enamel slab, over 24 h periods, for strains from Subjects A - G, n=4.**



No significant differences observed.

Fig. 5.7. **Mean (SD) change in total mineral loss (Δz - % vol min x μm) from bovine enamel slabs, incubated with sucrose and strains of Strep. mutans from Subjects A - G, n=6.**

Table 5.8. Lactate, acetate and total acid anion concentration (nmol/mg wet weight bacteria) over 24 h, with 5 % w/v sorbitol, for Strep. mutans strains from Subjects A - G, and Type Culture, n=2.

SUBJECT A
SORBITOL

		0	10m	30m	1h	5h	24h
Lactate	1	8.75	13.29	16.03	19.04	25.04	22.46
	2	5.50	7.13	10.56	13.43	16.76	20.82
	Mean (SD)	7.12 (2.30)	10.21 (4.36)	13.30 (3.87)	16.24 (3.97)	20.90 (5.85)	21.64 (1.16)
Acetate	1	4.64	7.08	11.36	14.43	25.87	31.29
	2	3.92	5.44	7.35	8.67	12.00	15.33
	Mean (SD)	4.28 (0.51)	6.26 (1.16)	9.36 (2.84)	11.55 (4.07)	18.94 (9.81)	23.31 (11.28)
Total acid	1	15.93	24.48	32.38	38.35	57.56	63.96
	2	12.96	16.43	22.65	27.21	33.04	41.86
	Mean (SD)	14.44 (2.10)	20.46 (5.69)	27.52 (6.88)	32.78 (7.88)	45.30 (17.34)	52.91 (15.63)

SUBJECT B
SORBITOL

		0	10m	30m	1h	5h	24h
Lactate	1	7.19	7.54	9.53	9.83	11.92	20.97
	2	5.12	5.86	7.53	7.31	10.12	17.42
	Mean (SD)	6.16 (1.46)	6.70 (1.19)	8.53 (1.41)	8.57 (1.78)	11.05 (1.32)	19.20 (2.51)
Acetate	1	3.43	4.41	5.20	6.17	7.63	10.64
	2	3.94	4.56	5.32	5.87	8.30	10.50
	Mean (SD)	3.68 (0.36)	4.48 (0.11)	5.26 (0.08)	6.02 (0.21)	7.97 (0.47)	10.57 (0.10)
Total acid	1	12.51	14.32	17.00	19.63	24.32	38.20
	2	10.10	11.62	14.48	15.61	20.76	31.78
	Mean (SD)	11.30 (1.70)	12.97 (1.91)	15.74 (1.78)	17.62 (2.84)	22.54 (2.52)	34.99 (4.54)

Table 5.8. cont.

SUBJECT D
SORBITOL

		0	10m	30m	1h	5h	24h
Lactate	1	30.12	21.78	19.39	22.44	14.76	59.65
	2	10.40	11.57	11.53	12.40	14.61	31.29
	Mean (SD)	20.26 (13.94)	16.68 (7.22)	15.46 (5.56)	17.42 (7.10)	14.68 (0.11)	45.47 (20.05)
Acetate	1	14.48	16.55	15.75	19.56	31.92	35.56
	2	9.98	10.01	9.54	10.42	13.49	20.02
	Mean (SD)	12.23 (3.18)	13.28 (4.62)	12.64 (4.39)	14.99 (6.46)	22.70 (13.03)	27.79 (10.99)
Total acid	1	55.47	48.48	44.82	51.66	54.32	111.98
	2	25.54	29.85	26.42	27.74	32.55	60.68
	Mean (SD)	40.50 (21.16)	39.16 (13.17)	35.62 (13.01)	39.70 (16.91)	43.44 (15.39)	86.33 (36.27)

Table 5.8. cont.

SUBJECT E SORBITOL		0	10m	30m	1h	5h	24h
Lactate	1	21.15	33.67	36.80	46.23	65.49	85.84
	2	25.43	32.57	32.94	46.90	65.08	81.68
	Mean (SD)	23.29 (3.03)	33.12 (0.78)	34.87 (2.73)	46.56 (0.47)	65.28 (0.29)	83.76 (2.94)
Acetate	1	6.29	15.26	16.27	19.82	26.73	46.88
	2	10.15	11.60	14.47	19.48	25.78	42.10
	Mean (SD)	8.22 (2.73)	13.43 (2.59)	15.37 (1.27)	19.65 (0.24)	26.26 (0.67)	44.49 (3.38)
Total acid	1	31.71	57.98	61.03	74.35	101.95	149.65
	2	40.54	48.82	54.97	74.99	97.67	139.31
	Mean (SD)	36.12 (6.24)	53.40 (6.48)	58.00 (4.28)	74.67 (0.45)	99.81 (3.03)	144.48 (7.31)

Table 5.8. cont.

SUBJECT F
SORBITOL

		0	10m	30m	1h	5h	24h
Lactate	1	25.44	22.75	27.86	32.71	43.89	65.90
	2	14.71	16.62	18.70	22.13	27.53	37.59
	Mean (SD)	20.08 (7.59)	19.68 (4.33)	23.28 (6.48)	27.42 (7.48)	35.71 (11.57)	51.74 (20.02)
Acetate	1	9.69	11.05	14.04	16.83	25.32	35.04
	2	7.22	8.83	11.94	13.22	21.33	29.17
	Mean (SD)	8.46 (1.75)	9.94 (1.57)	12.99 (1.48)	15.02 (2.55)	23.32 (2.82)	32.10 (4.15)
Total acid	1	44.80	42.30	51.05	58.15	77.72	111.57
	2	26.07	29.84	34.76	41.09	54.07	73.51
	Mean (SD)	35.44 (13.24)	36.07 (8.81)	42.90 (11.52)	49.62 (12.06)	65.90 (16.72)	92.54 (26.91)

Table 5.8. cont.

SUBJECT G
SORBITOL

		0	10m	30m	1h	5h	24h
Lactate	1	8.52	6.51	7.06	7.09	9.76	14.72
	2	7.34	7.01	8.05	8.58	11.13	15.96
	Mean (SD)	7.93 (0.83)	6.76 (0.35)	7.56 (0.70)	7.84 (1.05)	10.44 (0.97)	15.34 (0.88)
Acetate	1	10.44	12.77	13.25	12.60	21.26	28.13
	2	8.28	7.67	8.11	9.86	13.38	20.16
	Mean (SD)	9.36 (1.53)	10.22 (3.61)	10.68 (3.63)	11.23 (1.94)	17.32 (5.57)	24.14 (5.64)
Total acid	1	22.34	23.56	24.22	23.00	34.84	52.35
	2	19.98	18.66	20.45	24.66	31.97	46.67
	Mean (SD)	21.16 (1.67)	21.11 (3.46)	22.34 (2.66)	23.83 (1.17)	33.40 (2.03)	49.51 (4.02)

Table 5.8. cont.

TYPE CULTURE
SORBITOL

		0	10m	30m	1h	5h	24h
Lactate	1	7.58	8.44	10.88	10.05	15.46	22.02
	2	9.05	9.53	9.59	10.98	14.61	21.80
	Mean (SD)	8.32 (1.04)	8.98 (0.77)	10.24 (0.91)	10.52 (0.66)	15.04 (0.60)	21.91 (0.16)
Acetate	1	4.77	5.00	7.18	7.87	13.39	19.97
	2	4.63	5.29	7.54	9.01	13.83	20.09
	Mean (SD)	4.70 (0.10)	5.14 (0.20)	7.36 (0.25)	8.44 (0.81)	13.61 (0.31)	20.03 (0.08)
Total acid	1	15.20	16.72	21.39	21.08	32.51	46.09
	2	(16.49)	(17.98)	(18.61)	(22.64)	(31.58)	(46.05)
	Mean (SD)	15.84 (0.91)	17.35 (0.89)	20.00 (1.96)	21.86 (1.10)	32.04 (0.66)	46.07 (0.03)

Table 5.8. cont.

LACTATE

	0	10m	30m	1h	5h	24h
A	49	50	48	50	46	41
B	54	52	54	49	49	55
D	50	42	43	44	34	53
E	64	62	60	62	65	58
F	57	55	54	55	54	56
G	37	32	34	33	31	31
TC	52	52	51	48	47	48
Mean	52	49	49	49	46	49

ACETATE

	0	10m	30m	1h	5h	24h
A	30	30	34	35	42	44
B	32	34	33	34	35	30
D	30	34	35	38	52	32
E	23	25	25	26	26	31
F	24	28	30	30	35	35
G	44	48	48	47	52	49
TC	30	30	37	39	42	43
Mean	30	33	34	36	40	38

Table 5.9. Proportion of lactate and acetate, expressed as a percentage of the total acid, at each sampling time from 0 - 24 h, under sorbitol conditions, for Strep. mutans strains from Subjects A - G and Type Culture.

Table 5.10. pH measurements at each sampling time from 0 - 24 h, during incubation with 5 % w/v sorbitol, for Strep. mutans strains from Subjects A - G and Type Culture, n=4.

SUBJECT A SORBITOL

	0	10m	30m	1h	5h	24h
1	4.41	4.54	4.47	4.56	4.50	5.26
2	4.54	4.50	4.66	4.66	4.82	5.33
3	4.60	4.47	4.43	4.51	4.59	4.75
4	4.19	4.19	4.18	4.28	4.46	4.65
Mean (SD)	4.44 (0.18)	4.42 (0.16)	4.44 (0.19)	4.50 (0.16)	4.59 (0.16)	5.00 (0.35)

SUBJECT B SORBITOL

	0	10m	30m	1h	5h	24h
1	4.50	4.43	4.36	4.35	4.42	4.76
2	4.54	4.39	4.38	4.41	4.51	4.97
3	4.21	4.16	4.13	4.18	4.39	4.74
4	4.12	4.06	4.06	4.13	4.35	4.68
Mean (SD)	4.34 (0.21)	4.26 (0.18)	4.23 (0.16)	4.27 (0.13)	4.42 (0.07)	4.79 (0.13)

SUBJECT D SORBITOL

	0	10m	30m	1h	5h	24h
1	4.11	4.28	4.33	4.42	4.53	4.66
2	4.25	4.53	4.52	4.46	4.56	4.50
3	4.32	4.29	4.16	4.26	4.42	4.58
4	3.82	3.72	3.81	3.76	4.18	4.34
Mean (SD)	4.12 (0.22)	4.20 (0.34)	4.20 (0.30)	4.22 (0.32)	4.42 (0.17)	4.52 (0.14)

SUBJECT E
SORBITOL

	0	10m	30m	1h	5h	24h
1	3.91	3.92	3.90	3.93	4.15	4.55
2	3.90	4.02	4.00	4.01	4.23	4.56
3	3.48	3.55	3.53	3.77	3.80	4.48
4	3.60	3.69	3.74	3.60	3.87	4.16
Mean (SD)	3.72 (0.22)	3.80 (0.21)	3.79 (0.20)	3.83 (0.18)	4.01 (0.21	4.44 (0.19)

SUBJECT F
SORBITOL

	0	10m	30m	1h	5h	24h
1	3.93	4.12	4.12	4.11	4.26	4.47
2	4.19	4.19	4.30	4.48	4.67	5.26
3	3.55	3.62	3.64	3.74	4.11	4.24
4	3.70	3.67	3.73	3.86	4.17	4.38
Mean (SD)	3.84 (0.28)	3.90 (0.30)	3.95 (0.31)	4.05 (0.33)	4.30 (0.25)	4.59 (0.46)

SUBJECT G
SORBITOL

	0	10m	30m	1h	5h	24h
1	4.43	4.52	4.60	4.68	4.65	4.73
2	4.50	4.47	4.50	4.55	4.61	4.69
3	3.89	3.99	4.05	4.18	4.35	4.65
4	3.98	4.05	4.10	4.05	4.15	4.63
Mean (SD)	4.20 (0.31)	4.26 (0.28)	4.31 (0.28)	4.36 (0.30)	4.44 (0.23)	4.68 (0.04)

Table 5.10. cont.

TYPE CULTURE
SORBITOL

	0	10m	30m	1h	5h	24h
1	4.28	4.13	4.20	4.26	4.43	4.53
2	4.36	4.33	4.30	4.23	4.36	4.58
3	4.38	4.35	4.36	4.39	4.42	4.57
4	4.23	4.29	4.32	4.37	4.47	4.56
Mean (SD)	4.31 (0.07)	4.28 (0.10)	4.30 (0.07)	4.31 (0.08)	4.42 (0.04)	4.56 (0.02)

Table 5.10. cont.

CALCIUM RELEASE
SORBITOL

	1	2	3	4	Mean (SD)
A	2.42	0.62	0.82	1.10	1.24 (0.81)
B	1.33	0.42	1.96	3.88	1.90 (1.46)
D	2.15	1.61	2.19	5.56	2.88 (1.81)
E	3.43	3.97	8.20	3.50	4.78 (2.30)
F	1.70	0.69	1.65	3.16	1.80 (1.02)
G	1.82	3.10	1.33	1.83	2.02 (0.76)
TC	1.00	0.85	1.09	0.98	0.98 (0.10)

Table 5.11. Change in calcium concentration (mM) of slurry over 24 h periods following incubation of Strep. mutans, 5 % w/v sorbitol and bovine tooth slab, for Subjects A - G and Type Culture, n=4.

Δz SCORE
SORBITOL

	1	2	3	4	5	6	Mean (SD)
A	672	829	280	600	1113	-	699 (306)
B	230	815	0	139	229	266	280 (279)
D	209	97	426	517	235	445	322 (164)
E	1092	872	904	425	-	-	823 (283)
F	456	495	616	1033	712	511	637 (215)
G	54	35	40	0	-	-	32 (23)
TC	13	0	19	-	-	-	11 (10)

Table 5.12. Δz (% vol min x μm) of enamel sections obtained from bovine slabs incubated for 48 h with Strep. mutans and 5 % w/v sorbitol, for Subjects A - G and Type Culture, n=6.

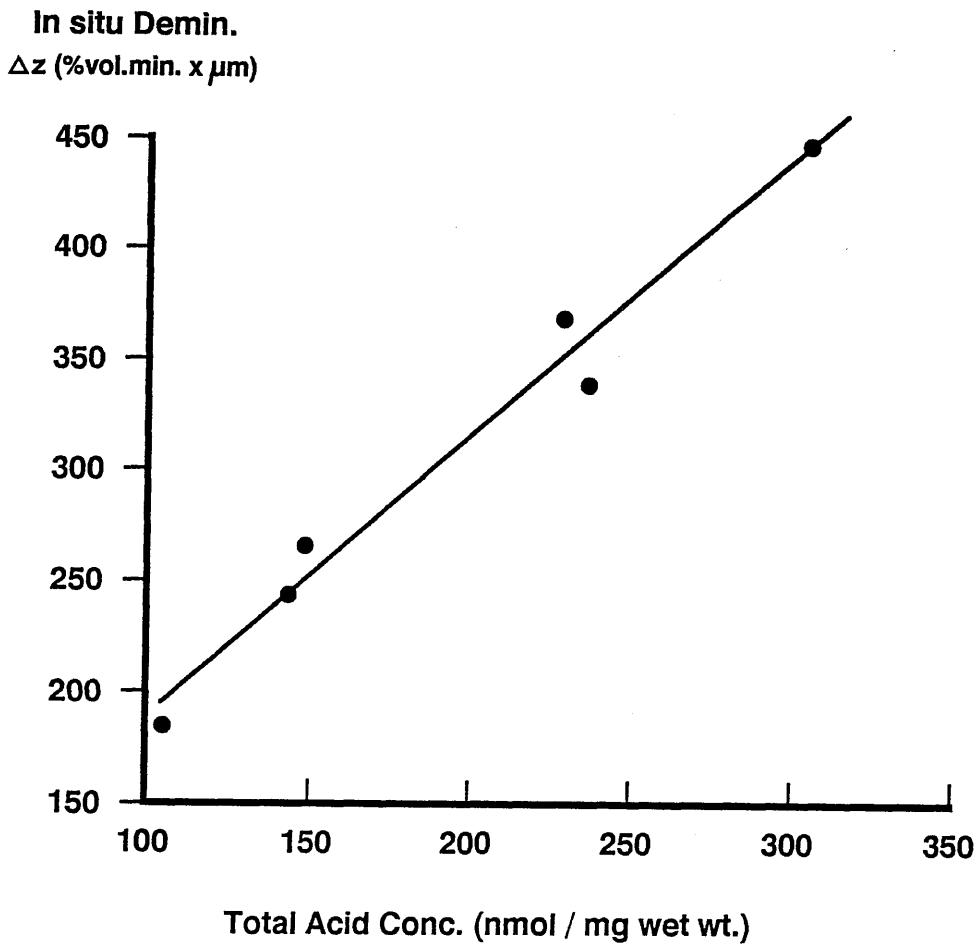


Fig. 5.8. Plot of mean total acid anion concentration (nmols per mg wet weight bacteria) at 24 h for each Strep. mutans strain against mean in situ Δz score (% vol min x μm) of the subject from whom the strain was isolated. r=0.99, n=6.

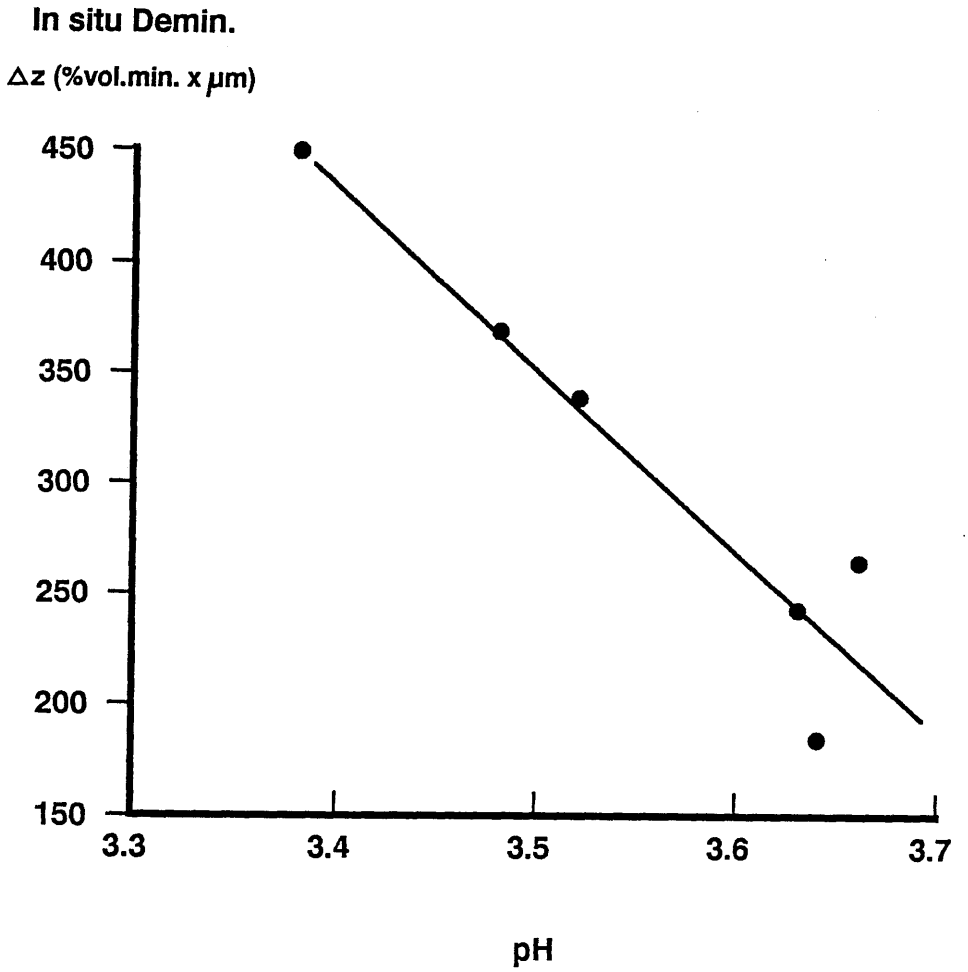


Fig. 5.9. Plot of mean minimum pH value for each Strep. mutans strain against mean in situ Δz score (% vol min x μm) of the subject from whom the strain was isolated. r=-0.95, n=6.

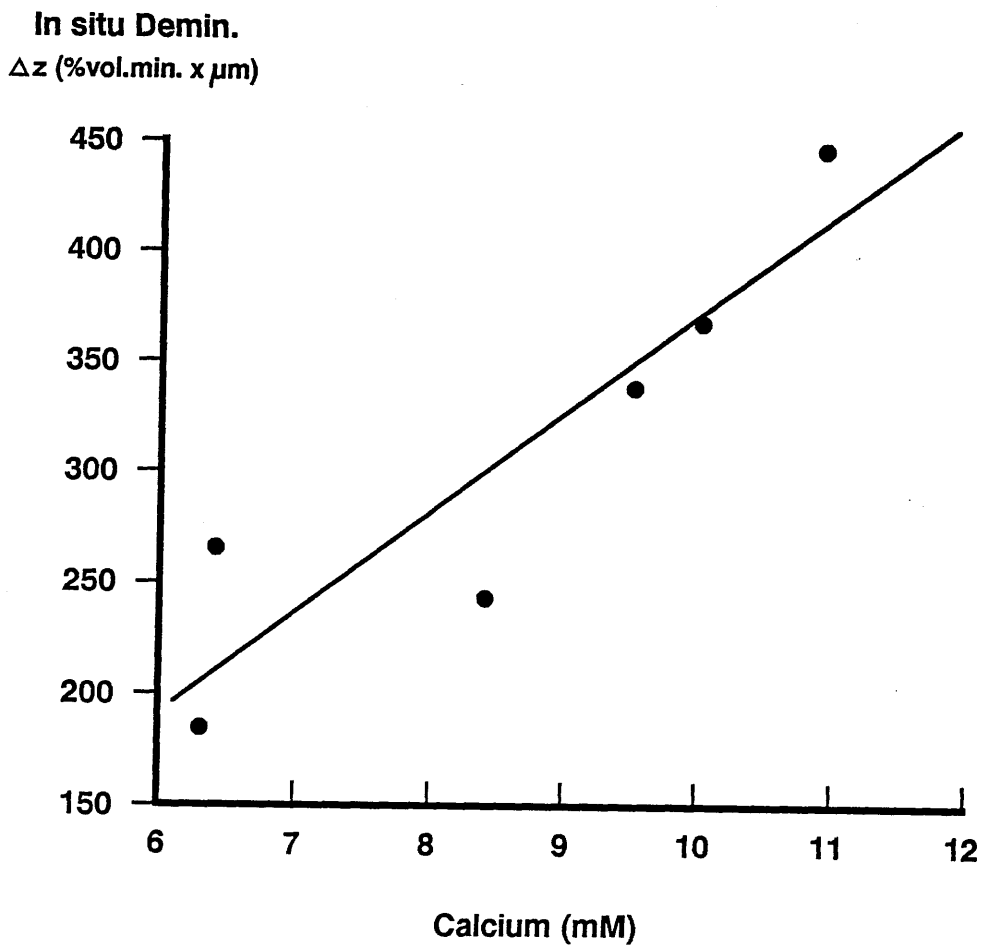


Fig. 5.10. Plot of mean change in calcium concentration (mM) at 24 h for each Strep. mutans strain against mean in situ Δz score (% vol min x μm) of the subject from whom the strain was isolated. r=0.91, n=6.

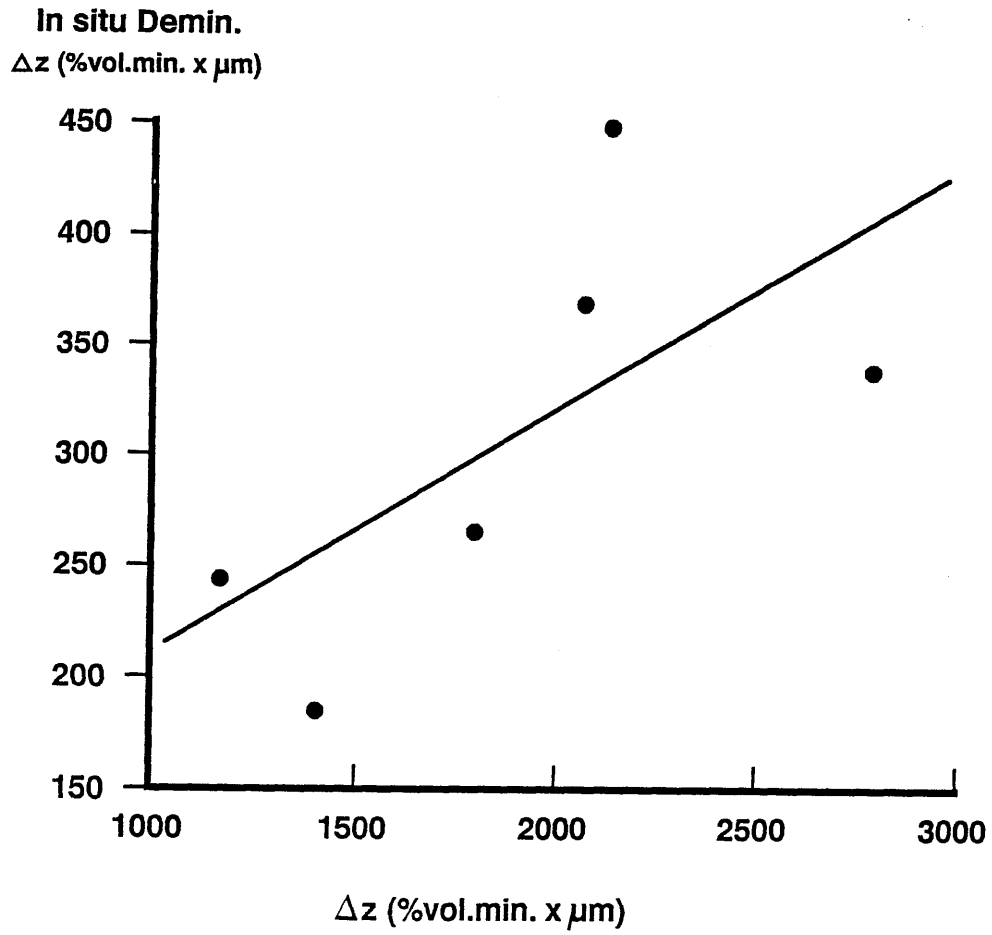


Fig. 5.11. Plot of mean bovine enamel Δz score (% vol min x μm) at 48 h for each Strep. mutans strain against mean in situ Δz score (% vol min x μm) of the subject from whom the strain was isolated. r=0.66, n=6.

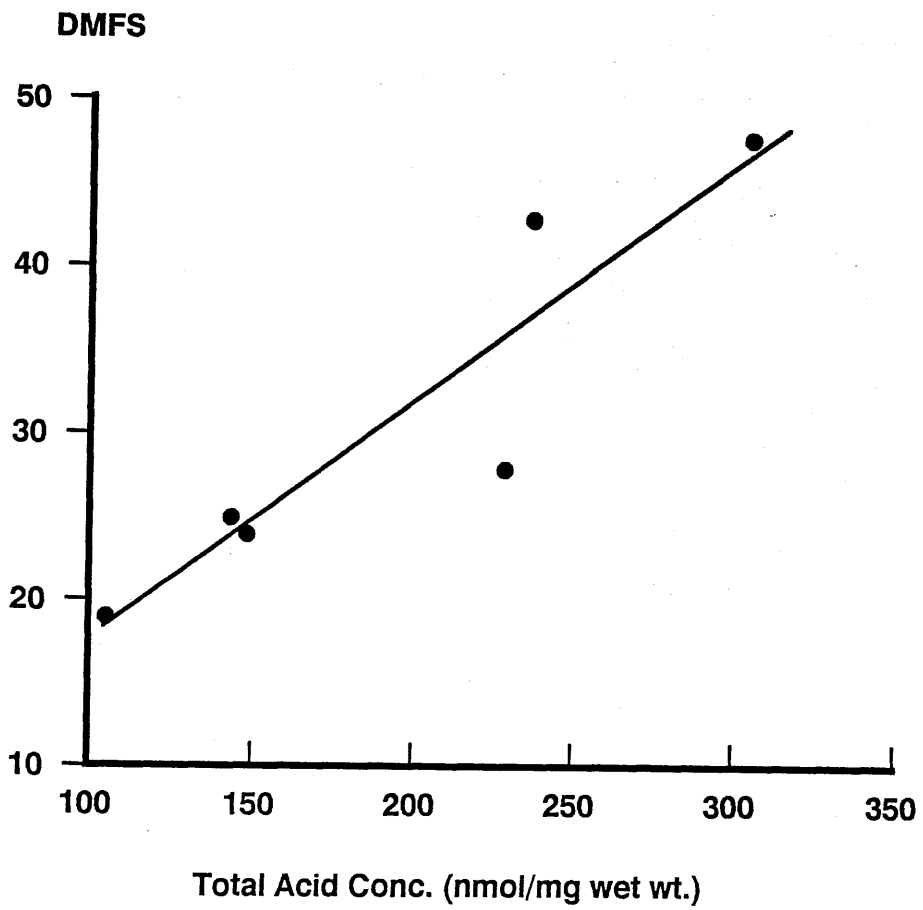


Fig. 5.12. Plot of mean total acid anion concentration (nmol per mg wet weight bacteria) at 24 h for each Strep. mutans strain against DMFS score of the subject from whom the strain was isolated. r=0.92, n=6.

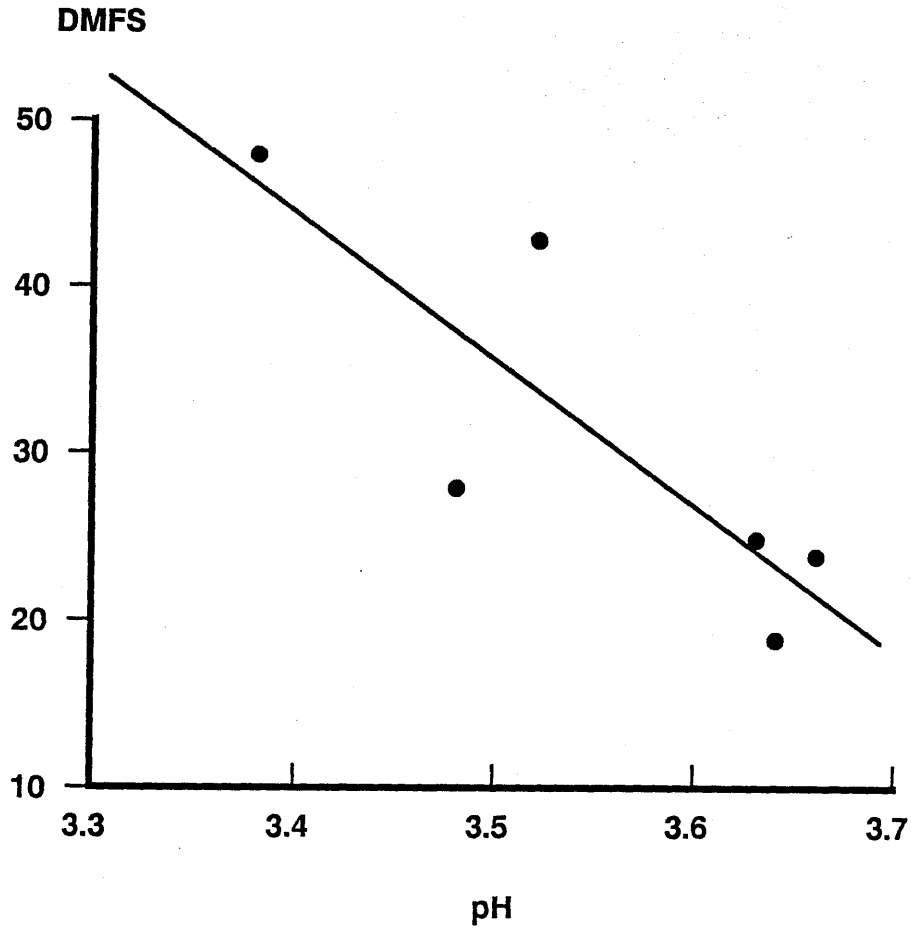


Fig. 5.13. Plot of mean minimum pH value for each Strep. mutans strain against DMFS score of the subject from whom the strain was isolated. r=-0.84, n=6.

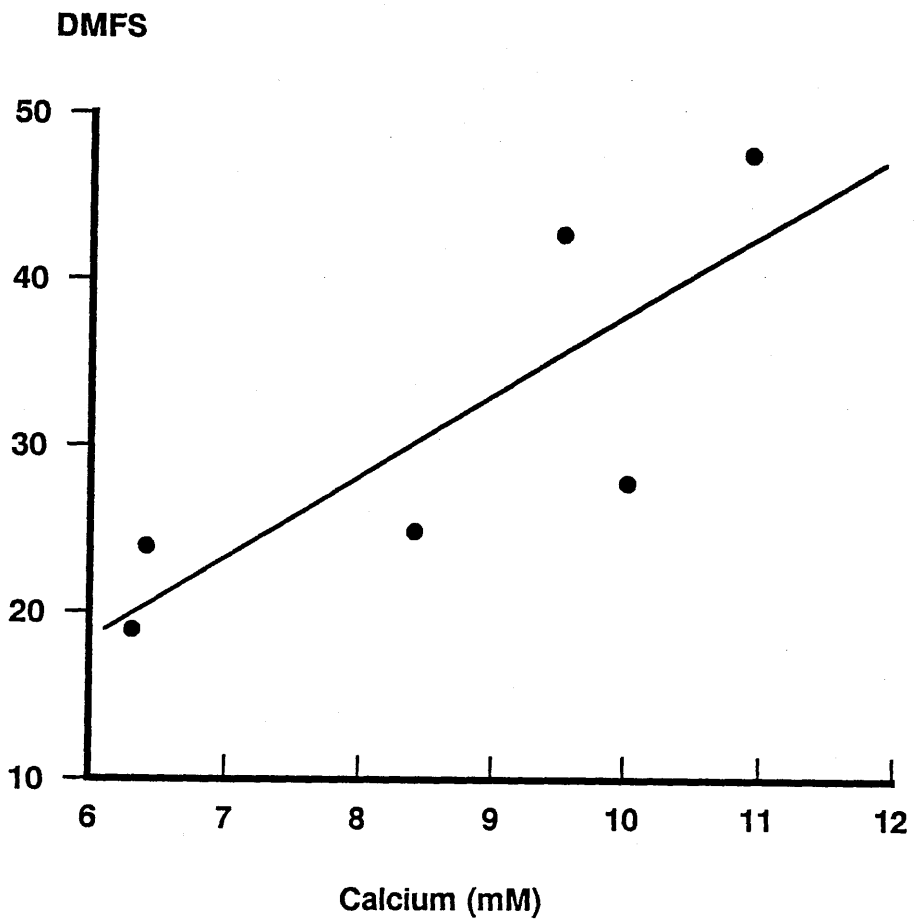


Fig. 5.14. Plot of mean change in calcium concentration (mM) at 24 h for each Strep. mutans strain against DMFS score of the subject from whom the strain was isolated. r=0.81, n=6.

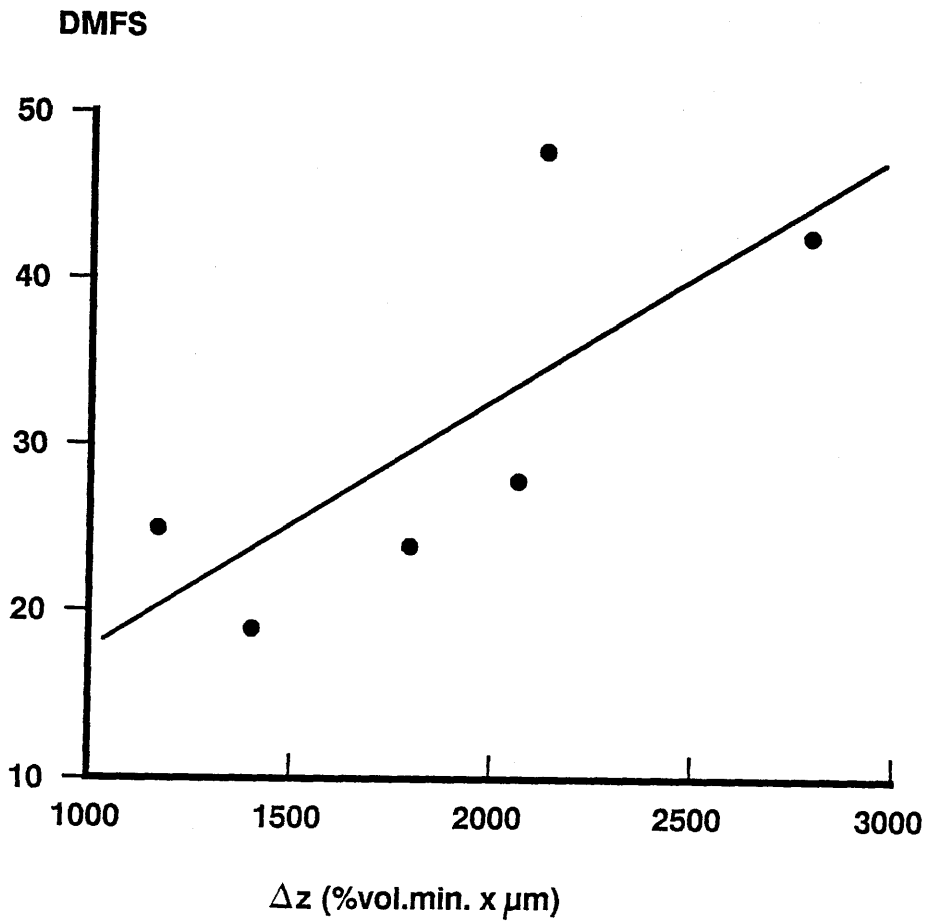


Fig. 5.15. Plot of mean bovine enamel Δz score (% vol min x μm) at 48 h for each Strep. mutans strain against DMFS score of the subject from whom the strain was isolated. r=0.75, n=6.

IN VITRO CARIOGENICITY TEST

	Total acid	min pH	ΔCa^{2+}	Δz slabs
DMFS	**	*	*	NS
In situ demineralisation	***	**	*	NS

SALIVARY CHARACTERISTICS

	Strep. mutans count	Lact. count	Flow rate	Buffering capacity
DMFS	NS	NS	NS	NS
In situ demineralisation	NS	NS	NS	NS

NS = Not significant ; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Table 5.13. Regression analysis of relationship between in vitro cariogenicity parameters and salivary characteristics, and DMFS and in situ demineralisation scores.

5.10. Discussion.

5.10.1. Use of Bovine Slabs in in vitro Demineralisation Study.

This in vitro study was based on the models described by Geddes and co-workers (1984) and Primrose and co-workers (1988). Abraded bovine enamel was used as it produces more rapid demineralisation than human enamel (Featherstone & Mellberg, 1981) and is perhaps less variable in susceptibility to acid attack. Furthermore, Primrose and co-workers reported that it was recommended by The Scientific Consensus Conference on Methods for Assessing the Cariogenic Potential of Food (1986), for use in in vitro cariogenicity tests.

5.10.2. Investigation of the Initial Low pH Values Observed During Preliminary in vitro Studies.

When assessing the acidogenic potential of different strains of Strep. mutans, the ideal situation is one in which the initial pH of each sample is around neutrality, as this ensures a similar baseline value for all strains. In addition, it allows the maximum pH fall of the system to be recorded during the experiment, thus demonstrating differences in ΔpH more clearly. However, in this study preliminary results with one strain (that isolated from Subject A) showed that the initial pH was around 4.5, and it was considered that the relatively acid pH may have been due to the delay incurred while weighing, during which time the bacteria may have been metabolising storage polysaccharides. A comparison of the standard procedure, and one where the bacteria were rewashed to remove any acids which had been produced prior to the addition of substrate, was therefore carried out. The full data relating to these experiments has not been tabulated in the Results section, but a précis of the results, and discussion of the possible factors influencing the initial pH values is given below.

The mean pH immediately after addition of the enamel slab and sucrose to the Strep. mutans, was 4.84 for cells which had been re-washed, compared to 4.54 for cells prepared in the standard manner. The mean concentration of total acid at baseline was 13.6 nmol/mg wet weight bacteria for re-washed cells, while the corresponding value for the control run was 16.7.

However, the initial pH in the re-washed samples was still below the 5.2 ± 0.3 reported by Primrose and co-workers (1988), using a type culture (NCTC 10449) of Strep. mutans, on which standardisation of the bacterial weights was not carried out and thus the delay incurred during the weighing procedure, in the current experiments, did not occur.

The low initial pH values were probably, in part, due to the fact that the strains were incubated overnight in an unbuffered chemically defined medium, as the pH of the Strep. mutans-containing broths, following the 18 hour incubation period, ranged from 4.50 to 4.53, with no increase found after washing with KCl. The sucrose solution, made by dissolving the sugar in double-distilled deionised water, also had a relatively low pH, ranging from 5.37 to 5.81.

While the rewashing procedure did reduce the acid concentration and increase the pH to some extent, compared to the samples in which the substrate was added immediately after the weighing procedure, it was considered that the difference produced by rewashing was not great enough to merit the inclusion of this time-consuming procedure in the experimental design. It was therefore omitted from the protocol used in subsequent studies. However, in retrospect, the inclusion of rewashing might have made more of a difference with some strains, particularly with D, E and F, where relatively large concentrations of lactate were found 15 s after the addition of sucrose to the vials, than with the strain used in this experiment.

Subsequent studies using Todd Hewitt broth as the overnight growth medium have supported the theory that the low pH was due to unbuffered culture, since, following incubation with this buffered medium and washing and weighing using the standard technique, the initial pH was considerably higher immediately after the addition of the sucrose substrate (range 5.8-6.2), but fell during the subsequent 24 hour experimental period.

5.10.3. Relative Cariogenic Potential of the Different Strep. mutans Strains.

The results showed that significant differences were found regarding the cariogenic potential of the six strains of Strep. mutans isolated from the

different individuals, with the type culture fitting into the low-intermediate category. Previous studies have reported differences in the cariogenic potential of various serotypes belonging to the Strep. mutans group (Kaufman et al., 1984) and differences in the presence and proportions of the various serotypes have been found between the plaques associated with caries-active and caries-free teeth (Kilian et al., 1979; Masuda et al., 1979). In the present study, serotyping of the Strep. mutans strains was not carried out, but biochemical profiles suggest that they all belonged to the biotype c group, according to the scheme of Shklair and Keene (1974).

Kaufman and co-workers (1984) compared the cariogenic potential of two serotypes of Strep. mutans and one strain of Strep. sanguis in an in vitro model involving human enamel, and found significantly different pH values and amounts of demineralisation produced by the two Strep. mutans strains. They suggested that this difference could have been due to differences in either the quantity of acid produced, or the ability of the strain to buffer its acid end products but, in the study, no qualitative or quantitative measurements of acids were made. Stosser and Kneist (1988), using a pH - stat system, found differences in the acidogenicity and acidurity of 20 strains of Strep. mutans, and inoculation of two of these strains produced different caries scores in rats.

In the present study, acids were quantified, and the significant differences seen amongst the strains with regard to demineralising potential and pH lowering capacity, appear to be due to differences in the ability of the strains to metabolise sucrose and produce acid at low pH, as some strains produced significantly greater amounts of acid than other strains under the test conditions.

The results for total acid concentration, minimum pH and calcium release, showed a strong association for the individual strains, with bacteria fitting into the same ranking groups for each of the three parameters studied. The Δz values obtained from the bovine slabs did not, however, exactly fit the same pattern, as strain E produced less change in Δz than strain F, which, according to the above criteria, fitted into the second rank group. This difference in ranking reflects the fact that microradiography and microdensitometry of sections prepared from the bovine slabs is probably

less sensitive than the other three methods of assessing cariogenic potential. This reduced sensitivity may be related to the fact that the use of slabs means that the same area of enamel cannot act as its own baseline control as in the case when sections are employed. Therefore, an area of enamel which was under the nail varnish coating during the experimental period had to be used as the control, with its Δz value being subtracted from the Δz value obtained from the exposed enamel window, in order to estimate the total mineral loss from the section of bovine enamel slab, during the experimental period. In addition, the sectioning and handgrinding procedures which were carried out post-experimentally may have resulted, on occasion, in some loss of the outer enamel surface, which had become partially demineralised during the experimental period.

The results obtained following incubation of the Strep. mutans strains with a 5 % sorbitol solution showed that significantly lower concentrations of acid and lower levels of demineralisation were produced than with the corresponding strains in sucrose. The ranking order of the different strains remained the same, with respect to total acid, minimum pH and calcium release, but the results of the Δz measurements were again more variable.

The total acid concentration at the various sampling times produced by the type strain and strains A, B and G, during incubation with sorbitol, were in a similar range to those reported by Primrose and co-workers (1988) using similar conditions. However, significantly higher concentrations of acid were produced by strains D, E and F, with the total acid at 24 hours for strain E being higher or similar to that produced by some of the other strains under sucrose conditions. The relative proportions of the different acids present in the samples under sorbitol conditions, were different from those obtained with sucrose incubation. Under the latter conditions, the predominant acid species was lactate, and a rise in proportion with time was seen, (62 - 80 %), whilst a decrease in proportion took place with regard to acetate which fell from 25 % at baseline to 12 % at 24 hours. With sorbitol incubation, lactate comprised approximately 50 % of the total acid on all sampling occasions, and the acetate proportion was higher than under sucrose conditions, ranging from 30 - 40 %, and showing an increase with time up to 5 hours.

The pH profiles associated with the two substrates were also different. Whereas a significant decrease in pH occurred with all strains of Strep. mutans under sucrose conditions following the initial reading, with sorbitol incubation, only strain B showed an appreciable drop in pH in the first 30 min of the experiment, while with the other strains the pH rose slightly with time.

In this study, the change in relative proportions of the acids under the two substrate conditions could be due to the different metabolic pathways used during sucrose or sorbitol excess. Under sucrose excess, the pyruvate formed is converted via the lactate dehydrogenase pathway to lactate, whereas incubation of Strep mutans cells with sorbitol may lead to larger amounts of acetate being formed. This could be due to metabolism of either storage polysaccharides, or sorbitol to pyruvate, which is further metabolised via the pyruvate formate-lyase pathway, forming acetate and formate as major end-products (Carlsson 1986).

Primrose and co-workers (1988) found no significant difference between the cariogenic potential of Strep. mutans type culture NCTC 10449 incubated with 5 % w/v sorbitol and the same type culture incubated with double-distilled and deionised water. The sorbitol control was therefore included in the present experiment to determine whether variation existed in the quantity and utilisation of storage polysaccharides, among individual strains. However, although large differences were found in the concentrations of acids produced by different strains, the experiments would have to be repeated with incubation in double-distilled and deionised water alone, to determine whether some of the acid production was due to metabolism of the sorbitol, and whether variation in this ability existed amongst the strains.

5.10.4 Relationship Between in vitro Cariogenicity Test Parameters and Natural and Experimental Caries Experience of the Individuals.

The cariogenic potential of each strain was determined by assessing both its acidogenic and demineralising potential. A comparison of the ranking order of the in vitro cariogenic potential of the different Strep. mutans strains with the natural and experimental caries experience of the individuals from whom the strains were isolated, has not previously been

performed. This study showed a strong correlation between the in vitro results and the DMFS and in situ demineralisation scores of the subjects. This was particularly evident regarding total acid concentration at 24 hours and the minimum pH values obtained during the incubation period. The change in calcium concentration of the system, which was one of the methods of assessing the mineral loss from the bovine enamel slabs, also correlated with both the DMFS and in situ Δz scores associated with each subject. While there was a trend towards a relationship between the Δz values of the bovine enamel and the natural and experimental caries levels, in neither case did this reach significant levels and, as mentioned previously, this could be due to the fact that this may be a less sensitive method of assessing mineral loss.

When considering the levels of in situ demineralisation, for the purposes of this experiment, only the results from the normal plaque and sucrose plaque conditions were taken into account, as implantation of Strep. mutans was not performed in two of the six subjects whose Strep. mutans strains were investigated in this in vitro study.

No significant correlations were seen between any of the salivary characteristics, investigated in Chapter IV, and the caries experience of the individuals. This is in contrast to a number of clinical studies involving children, in which significant relationships between salivary levels of Strep. mutans and Lactobacillus spp. and present or future caries activity have been found (Klock & Krasse, 1977; Crossner, 1981; Stecksén-Blicks, 1985). However, in these studies, a very much larger sample size was used, and the predictive ability on a subject basis was poor, with the sensitivity and specificity of the test for individuals being 0.50 and 0.80 respectively in the study by Crossner. Similarly, Russell (1987) found that the use of microbiological tests enabled accurate prediction of caries increment on a high, medium or low group basis, in less than 50 % of individuals.

In the present study, the best association between the initial screening tests and the in situ experimental demineralisation level was the past caries experience of the natural dentition of the individual ($p < 0.05$). Differences in the usefulness of this parameter as a predictor of future caries activity have been found in different studies, with some finding this

to be better than any other screening tests (Klock & Krasse, 1979; Honkala et al., 1984), while others have found it to be less sensitive than microbial counts (Crossner, 1981).

In the present investigation, the cariogenic potential of the various strains was based mainly on their ability to survive and produce acid at low pH. The viable counts of Strep. mutans at the different sampling times were not monitored in this study, but this should be incorporated into future studies to determine whether differences are found, between strains, in the numbers of viable bacteria, and to calculate the 24 hour count as a proportion of the initial count.

Another factor which may be important regarding the cariogenic potential of a Strep. mutans strain is its ability to adhere to the tooth surface. Differences in the potential of the strains to adhere may have been partly responsible for the differences in levels of colonisation seen following implantation of the strains in the appliance studies. This may account for the fact that less good correlation was found between the levels of in situ demineralisation following implantation of Strep. mutans and the results of in vitro cariogenicity tests using the same strains, than the correlation between in vitro results and the demineralisation seen, in the appliance model, with normal and sucrose plaques.

In this preliminary study, only one strain from each of the six volunteers was used. Although all of the Strep. mutans colonies isolated from each individual which were tested using the biochemical scheme of Shklair and Keene (1974) belonged to biotype c, in future studies, multiple strains from each subject should be investigated to determine whether differences exist in the cariogenic potential of different strains within an individual. Further, the reproducibility of the results in relation to each strain should be tested, as some variation was seen among the four experimental runs, for each strain involved in this experiment, particularly with regard to Δz values.

The results of this study suggest that, in addition to actual numbers of Strep. mutans present, the cariogenic potential of the individual strains is relevant in determining whether or not demineralisation will occur. Thus, investigation of cariogenic potential and the ability of the organism to

adhere, should perhaps be incorporated in future caries activity tests and, although the multifactorial nature of the carious process means that the relevance of other factors should also be considered, this model may be useful as one of several screening tests in assessing the caries risk of an individual.

5.11. Conclusions.

Significant differences were found in cariogenic potential of the Strep. mutans strains isolated from six of the individuals involved in the appliance study described in Chapter IV. While there was no significant relationship between the salivary characteristics and the subject's natural and experimental caries levels, the in vitro cariogenic potential of each strain correlated significantly with the DMFS score and in situ demineralisation experienced by the individual from whom the strain was isolated.

Whilst this preliminary study involved only six adult volunteers, and was carried out using only one strain of Strep. mutans from each subject, the results obtained suggest that further work, using a much larger number of subjects and multiple strains from each individual, should be undertaken. In addition, such an investigation should be carried out using a child population. The preliminary work on this small sample suggests that the model may have potential for development as an additional screening test, for the detection of high caries risk individuals.

CHAPTER VI.

IN SITU EARLY COLONISATION STUDY.

6.1. Introduction.

Several studies have characterised the human microflora during the early stages of colonisation of teeth (Socransky et al., 1977; Syed & Loesche, 1978; Nyvad & Kilian, 1987) and have found that Streptococcus spp. dominate the plaque, with a minor contribution from Actinomyces spp. However, much of the information available on early plaque development has been obtained from ultrastructural studies (Saxton, 1973; Nyvad & Ferjerskov, 1987), light microscopic studies of Gram staining and morphological groups (Theilade & Theilade, 1970), investigation of the developing flora on artificial supporting structures such as plastic films or Mylar strips (Ronstrom et al., 1977; Theilade et al., 1982) or from studies involving fissure inserts (Theilade et al., 1974; Svanberg & Loesche, 1977), whilst only a relatively small number of cultural studies of early smooth surface supra-gingival plaque have been carried out (Socransky et al., 1977; Takamori et al., 1978; Moore et al., 1982; Nyvad & Kilian, 1987).

An investigation was therefore undertaken to ascertain whether the appliance model could be utilised to provide additional data on the compositional and numerical changes taking place during the early stages of plaque development on enamel surfaces. The study was conducted in a longitudinal manner and was designed to allow -

i) the identification and enumeration of the developing microflora during 48 hour periods, and

ii) the assessment of the effects of regular sucrose applications and the implantation of Strep. mutans on plaque development.

In addition, the test was carried out over periods of three weeks, with plaque sampling, for analysis as above, carried out at 2, 7 and 21 days.

6.2. Experimental Methods.

Two Day Study.

Enamel slabs of known surface area were prepared as described in Section 2.3.7. Twelve slabs were mounted on to each trough area of the appliance (Fig. 6.1.), and the device was worn for periods of 48 hours, during which time the subject's normal diet was maintained. Three volunteers were involved in this study. One individual participated in the three experimental protocols, outlined in Section 4.3.1. carried out over three separate 48 hour periods, whilst the other two subjects were each involved in a single two-day experiment, during which time a 10 % w/v sucrose solution was applied to one side, as described in Section 3.4.2., whilst the other trough area remained untreated.

Plaque samples were collected from the enamel slabs after 2, 6, 10, 24, 30 and 48 hours as described in Section 2.5.2. On each occasion, plaque was harvested from four slabs - two on each side. The samples were collected in this way in an attempt to minimise any effect that the position of each slab in the trough area may have had on the composition of the plaque microflora. Each sample was dispersed in 1 ml ABB, diluted and inoculated on to blood agar, MSB and Rogosa SL agar plates as described in 2.5.4. After appropriate incubation, also described in Section 2.5.4., the plaque composition was determined and the isolates expressed both as a percentage of the total colony forming units, and as a count per mm^2 of enamel surface as described in Section 2.5.5.

Twenty-one Day Study.

Nine enamel slabs were mounted on to each side of the removable appliance of one subject and the device was then worn for periods of three weeks. Plaque samples were collected from three slabs on each side at 2 days, 7 days and 3 weeks. Three separate experimental runs were carried out on the one subject, during which time the three experimental protocols outlined in Section 4.3.1. were followed, giving a total of 6 slabs per sampling time for each of the NP, SP and SPM conditions. The plaque samples were processed as described above, and

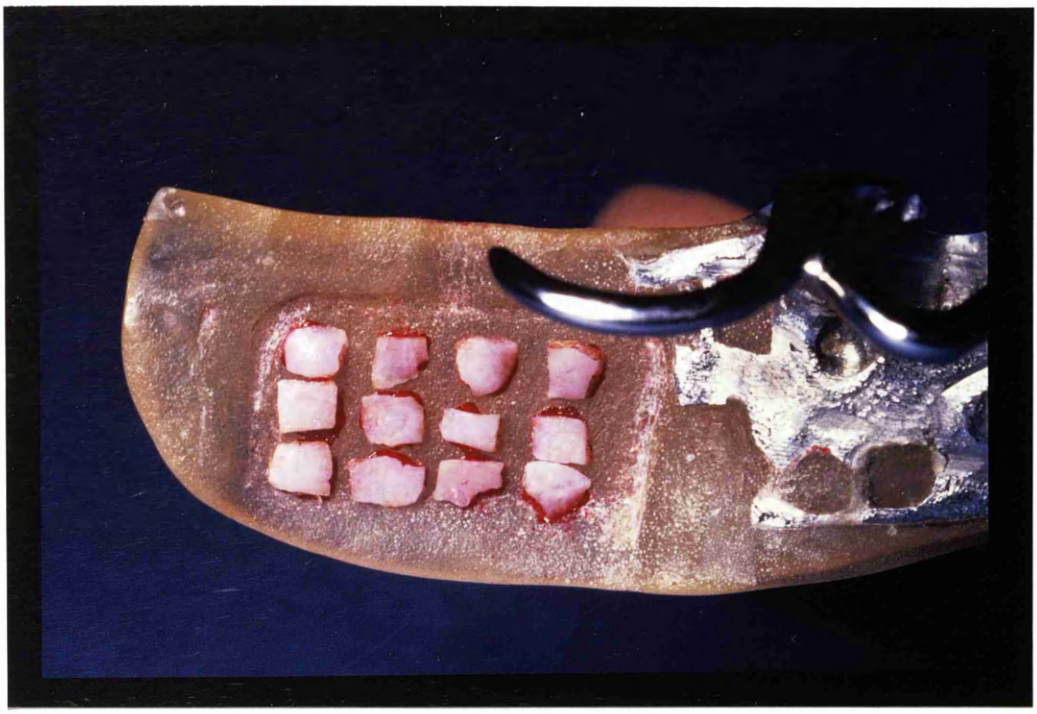


Fig. 6.1. Twelve enamel slabs mounted on trough area of appliance.

the microbial composition determined, both by proportional and absolute counts.

Data Handling with Regard to Absolute Counts.

The absolute counts were expressed in decimal log form, and for each sample, half the minimum detection value of the plate from which the count was obtained was added to the count of each organism under study, before calculating its count per mm^2 enamel surface and then log transforming (see Section 2.8.).

As Haemophilus spp. were not detected in a large proportion of the samples obtained from Subject A, in both the 2-day and 21-day experiments, the log count which would have been obtained using the above method would, on almost all occasions in normal plaque, have represented merely the value of half the detection limit on the non-selective plate from which the counts were being obtained. Therefore they would not have reflected the true situation. For this reason, absolute counts for Haemophilus spp. have not been given.

Similarly, Lactobacillus spp. was very rarely isolated in the 2-day study. Although the estimation of half the detection limit was made on the neat selective plates, and therefore was a much smaller value than that obtained from the non-selective plates, as Lactobacillus spp. was isolated from only a few samples, this estimation was not thought to be appropriate. The organism was therefore excluded from the Tables in which absolute counts were given, for the 2-day study.

However, one of the aims of this experiment was to assess the effect of the implantation of Strep. mutans on the composition of the microflora and the viable numbers of this organism isolated. Although Strep. mutans was not isolated from all samples, it was isolated from most samples obtained under SPM conditions and, as a selective medium was used, the estimated value for the neat plates on which the organism was not detected was relatively small. Therefore absolute counts are given for Strep. mutans as this allows assessment of the change, with time, in count of the organism per mm^2 enamel surface, following implantation.

Absolute counts were calculated for the remainder of the predominant organisms, but have not been given for the organisms which were only occasionally isolated, for reasons indicated above.

6.3. Results.

As plaque samples in the 2-day experimental runs were obtained under all three experimental protocols only for Subject A, to allow assessment of the effect of SPM conditions on plaque microflora, the results for this subject are considered separately. Similarly, the three-week runs were performed only by Subject A and so, by combining the data from the two experiments for this subject, the effect of time from 2 hours to 3 weeks could be investigated. In the early plaque studies, combined results for the three subjects have also been given, to allow comparison of the both normal and sucrose plaque microflora at different sampling times.

6.3.1. Microbial Composition of Plaque Samples Overlying Enamel Slabs, During Two Day Experimental Periods.

Subject A.

The predominant cultivable plaque microflora, expressed as a percentage of the total flora, for Subject A, under each treatment condition, is shown in Table 6.1., and the absolute counts of the isolates are given in Table 6.2. The statistical analyses of variance for these results are given in Table 6.3. and 6.4., and the latter shows that a highly significant increase in total microbial count occurred with time.

Positive cocci predominated in all plaque samples, ranging from 63.2 % to 93.9 %, with no significant effect of time or treatment condition with regard to mean percentage count (see Table 6.3). However, a highly significant increase in absolute count occurred with time ($p < 0.001$), in each treatment group. The absolute count of positive cocci was significantly higher in SP than in NP samples ($p < 0.05$), with the values for SPM samples being intermediate. As positive cocci comprised the majority of organisms isolated from each sample, the same pattern and level of significance is seen for effect of treatment on total microbial counts.

Strep. oralis accounted for the vast majority of the streptococci isolated under all treatment conditions, at most sampling times, rising in proportion up to 30 hours, but then falling slightly in the final sample, with the effect of time being significant at the 1 % level. Similarly, the absolute counts rose, in general, with time ($p < 0.001$) and only in sucrose plaque did the numbers of this organism fall at 48 hours. No treatment effect was seen with regard to percentage count, but the absolute count of Strep. oralis was higher in sucrose than normal plaques ($p < 0.05$). Strep. mutans was not isolated from any sample of the normal or sucrose plaques, but under SPM conditions comprised 11.0 and 28.1 % of the total microflora at 2 and 6 hours, respectively, before falling to undetectable levels at 10 hours. The organism was then isolated at 0.1 % of the total microflora in all SPM samples from 24 to 48 hours. However the absolute count rose from 1.10 $\log_{10}$ cfu per mm^2 enamel surface, at 2 h, to 2.73 at 24 h, in SPM samples. Although the increase was not seen in NP and SP samples, the rise in absolute count with time was significant at the 5 % level. Although no significant treatment effect was seen with regard to percentage counts, the absolute numbers of Strep. mutans were significantly higher ($p < 0.001$) in the SPM samples, compared to plaques where the organism had not been inoculated.

Strep. sanguis comprised a very variable proportion of the microflora in all treatment conditions, ranging from Not Detectable to 46.4 %, but was significantly higher in normal plaque than in samples obtained under the other protocols ($p < 0.01$), and was generally higher at the 2 and 48 hour sampling times (again, $p < 0.01$). However, despite a slight fall at 6 hours under NP and SP conditions, the absolute count of Strep. sanguis showed a highly significant increase ($p < 0.001$) with time.

Positive bacilli comprised a mean of 23.4 and 20.0 % in the 2 hour samples from normal and sucrose plaques, respectively, but only 1.8 % in the 2 h samples obtained under SPM conditions. In the last group, positive bacilli always accounted for less than 6 % of the total flora. Under all treatment conditions, positive bacilli consisted almost entirely of Actinomyces spp. In normal and sucrose plaques, the proportion of actinomyces fell between 2 and 10 hours, but reference to the absolute counts shows that the number of organisms remained fairly static over this period, before rising 1000-fold up to 48 hours.

The percentage distribution of A. odontolyticus and the catalase positive and negative divisions of A. viscosus / naeslundii, isolated from plaque obtained from Subject A under each treatment condition, at all sampling times is shown in Table 6.5. A. odontolyticus was isolated at only one sampling time (24 hours) under normal plaque conditions, but was isolated at all sampling times from sucrose plaque, and all but two sampling times from SPM. Catalase positive members of the A. viscosus / naeslundii group were isolated on only four of 18 sampling times with no treatment effect being seen, but this organism was never isolated from samples obtained at 24 - 48 hours. The catalase negative division of this group also tended to be isolated only from the early samples, and in the two hour samples under NP and SP conditions, comprised 14.9 and 8.9 % respectively of the total plaque flora.

Lactobacillus spp. were not isolated from any sample under the three treatment conditions.

Veillonella spp. comprised almost the total negative cocci isolated, and the mean percentage count showed no obvious trend in relation to time or treatment conditions, but the absolute counts showed a highly significant increase with time ($p < 0.001$, see Table 6.4.), and the numbers were generally higher in the sucrose plaque samples compared to normal plaque ($p < 0.05$).

Negative bacilli comprised a mean of between 6.5 and 13.0 % of the total microflora for most of the NP and SP samples, but accounted for only 1.9 % of the NP flora at 2 hours, and 2.4 % of the SP flora at 30 and 48 hours. Under SPM conditions, the proportion of negative bacilli decreased with time, from 9.0 % at two hours to 2.1 % at 48 hours, but overall, no significant time or treatment effect was seen. However, the absolute count of negative bacilli increased with time in all treatment groups ($p < 0.001$), with no significant difference between treatment conditions.

Haemophilus spp. were not detected from any sample under NP conditions, but were isolated from at least one sample of SP and SPM plaque at each sampling time, with the effect of the sucrose addition being significant at the 1 % level.

Bacteroides spp. accounted for 1.9 % of the 2 hour plaque under NP conditions, but thereafter ranged from 7.8 to 12.2 % in this treatment group. This was significantly higher ($p < 0.001$) than the proportions obtained from SP and SPM plaques. However, with reference to absolute counts, no significant treatment effect was seen, but the numbers of the organism increased with time, in all treatment groups ($p < 0.001$).

Three Subjects.

The percentage distribution of the predominant cultivable flora isolated at each sampling time, for three subjects combined, under NP and SP conditions is shown in Table 6.6., and a comprehensive summary of all the organisms isolated is given in Table 6.7. The absolute counts of the predominant organisms are shown in Table 6.8., and the statistical analyses are given in Tables 6.9. and 6.10.

As was found with the results from Subject A alone, the positive cocci comprised a very high proportion of the plaque microflora at all sampling times under both treatment conditions, and ranged from 66.5 - 88.2 %. The proportion of this morphological group tended to rise in both groups from the initial value but then fell again after 30 hours, and the difference in proportions with time was significant at the 5 % level (see Table 6.9.). The absolute count of positive cocci rose significantly with time ($p < 0.001$), and was also significantly greater under sucrose conditions compared to normal plaque ($p < 0.01$), with the maximum difference being seen at 30 hours, although the results for NP and SP samples were similar at 48 hours.

The total count of organisms isolated paralleled results for the positive cocci, rising with time ($p < 0.001$), and being higher in the samples exposed to extra-oral sucrose applications ($p < 0.01$). Similarly, the absolute count of all groups, genera and species under study rose with time, including Strep. mutans which comprised only a very small proportion of the total microflora, and with respect to percentage count, showed no relationship with time. For both percentage and absolute counts, no significant treatment effect was seen regarding Strep. mutans.

Strep. oralis was the most abundant species on all sampling occasions, and, as with positive cocci as a whole, rose in proportion following the initial sample to a maximum at 30 hours, and fell thereafter. This time effect was significant at the 0.1 % level. The absolute count of Strep. oralis, other than the 48 hour sample under SP conditions, rose with time ($p < 0.001$). The proportion of Strep. oralis was slightly higher in sucrose plaque samples (not significant), but a more marked difference was seen when comparing absolute counts (significant at the 1 % level).

Strep. sanguis showed the opposite trend, with regard to proportions, being highest at 2 and 48 hours ($p < 0.05$), and more abundant in the NP than SP samples ($p < 0.05$). However, no significant treatment effect was seen with regard to absolute counts, which rose with time in both treatment groups ($p < 0.001$).

The mean proportions of Strep. salivarius (seen in Table 6.7.) under NP and SP conditions at 2 hours were 5.2 and 10.5 %, respectively, and therefter a fall took place to undetectable levels at 24 hours, in both treatment groups. However, the organism was detected in low proportion at 30 and 48 hours, comprising less than 1 % of the total microflora at the latter sampling time.

At 2 hours under normal plaque conditions, members of the Micrococcus / Staphylococcus group accounted for a mean level of 8.3 % of the total microflora, but in all subsequent samples in this treatment group, and in all sucrose plaques they were either undetected or present in very low proportions.

The mean proportion of positive bacilli fell from the initial value, before rising again in the later samples (significant at the 5 % level). No significant treatment effect was seen in the proportions of positive bacilli, but the absolute counts were significantly higher under SP conditions ($p < 0.01$), with the greatest difference being found at 30 hours. The absolute count of positive bacilli fell slightly from 2 to 6 hours, but rose thereafter.

Actinomyces spp. followed the pattern described for positive bacilli, with the lowest values being found at 10 hours in both cases, although the

proportions for SP were slightly higher. The effects of time and treatment on mean percentage count were both significant at the 5 % level. The absolute counts of this genus were again higher in sucrose than in normal plaques (significant at the 1 % level), and under both treatment conditions, again followed the pattern with time described for positive bacilli. At the species level, A. odontolyticus was present at all sampling times from SP plaque, and all but one time (10 hours) from NP plaque. Overall, the proportion of the species decreased after the 2 hour sample, but rose again at 24 hours. The percentage counts were generally higher in the SP samples. The catalase positive division of A. viscosus / naeslundii was isolated at only four sampling times for the two treatment conditions combined, and the mean result never comprised more than 3 % of the total microflora. The catalase negative division was isolated at more sampling times, and was present in the highest proportion at 2 and 6 hours for both NP and SP, where the counts ranged from 2.5 to 5.0 %.

Although the lowest proportion of negative cocci was found at 30 hours, for both NP and SP conditions, the proportion and absolute counts, in general rose with time (significant at the 1 % and 0.1 % levels, respectively). The values obtained from SP samples were significantly higher than from the samples produced under NP conditions. In general, Veillonella spp. paralleled the results for negative cocci, but in several samples from 24 hours on, Neisseria spp. accounted for a greater proportion of the total flora than did veillonella.

The proportion of negative bacilli, under NP conditions, remained stable with time at around 7 - 10 %, while in sucrose plaque the percentage count of negative bacilli decreased overall, with time, from 9.0 to 2.6 %. Although, for the two treatments combined, no significant time effect was seen with regard to mean percentage counts, in general the absolute count rose in both normal and sucrose plaques with time, and this was significant at the 0.01 % level. The number of negative bacilli was higher in sucrose than in normal plaques ($p < 0.05$), with the greatest difference again being seen at 30 hours.

Haemophilus sp. was isolated from all but one sampling time under NP conditions and at all sampling times from sucrose plaque. The proportion

of Haemophilus sp. showed no pattern with time, but was generally slightly higher in the NP samples (significant at the 5 % level). Similarly, the proportion of Bacteroides spp. was higher under NP conditions ($p < 0.01$) but no time effect was seen. However, as with all other organisms, the absolute count generally rose with time ($p < 0.001$), although the 48 hour value in sucrose plaque was lower than the 30 hour result. No significant treatment effect was seen with regard to absolute counts.

A histogram summarising the percentage distribution of Gram positive and negative cocci and bacilli for all subjects, under both treatment conditions, at each sampling time, is shown in Fig. 6.2. and a histogram showing the mean total counts obtained from each treatment condition, at each sampling time, for the three subjects combined is shown in Fig. 6.3. In Figs. 6.4. and 6.5. the changes with time of the positive and negative coccal and bacillary counts, and the total counts, under NP and SP conditions respectively are indicated, and it can be seen that the total counts closely parallel the Gram positive coccal counts on both graphs.

The mean differences in $\log_{10}$ counts per mm^2 enamel surface, between SP and NP plaques, at each sampling time are shown in Fig. 6.6. The NP counts for each Gram staining and morphological group, and the total counts, have been subtracted from the corresponding SP counts, and the resultant values plotted. It can be seen that, with the exception of the 48 hour negative bacillary result, on every occasion, the calculated value is positive, indicating that higher counts were obtained from the plaques exposed to extra-oral sucrose applications.

6.3.2. Microbial Composition of Plaque Samples Overlying Enamel Slabs, During Twenty-One Day Experimental Periods.

The percentage distribution of the predominant cultivable microflora isolated from one subject from 2, 7 and 21-day plaque samples, under NP, SP and SPM conditions, is shown in Table 6.11., and a comprehensive summary of all organisms isolated is shown in Table 6.12. The counts of the predominant microflora at each sampling time is shown in Table 6.13., and the statistical analyses of variance of the proportional and absolute counts are shown in Tables 6.14. and 6.15., respectively.

The mean proportion of positive cocci decreased in all treatment groups with time, the 2-day results ranging from 76.3 - 92.4 %, compared to the range of 25.6 - 46.0 % for the 21-day samples. This change was highly significant ($p < 0.001$ - see Table 6.14.) This relationship with time was mainly attributable to Strep. oralis, whose results followed a similar pattern of equal significance. However, reference to absolute counts of the microflora (Table 6.13.) shows that, overall, no significant change in Strep. oralis occurred with time. No treatment effect was seen for this organism, for either proportional or absolute counts. These results were paralleled by the results for positive cocci, and the total microbial counts, where no relationship with time or treatment conditions was seen.

The mean proportion of Strep. sanguis increased with time under normal plaque conditions but, in both SP and SPM, plaques showed a slight increase from 2 to 7 days, followed by a marked fall in proportion. Overall, there was no significant time effect with reference to proportional counts, but the values obtained from SP samples were significantly higher than those from NP and SPM ($p < 0.01$). This treatment effect was only significant at the 5 % level for absolute counts, but here, a significant change in numbers with time was seen ($p < 0.05$), with the counts rising at seven days, and falling again slightly in the final samples.

Strep. mutans was essentially unchanged in proportion with time in NP and SP conditions, but showed a marked rise in mean percentage count from 1.6 % at 2 days to 18.0 % at 21 days in samples where the organism had been implanted. A significant treatment effect ($p < 0.001$), occurred with Strep. mutans, which was considerably higher at all sampling times under SPM conditions compared to the other two treatment groups. A similar difference between SPM, and the plaques where the organism was not inoculated, was seen with reference to absolute counts (significant at the 0.1 % level) but, unlike the proportional results where no significant change with time occurred, the absolute counts rose between 2 and 7 days under all treatment conditions and remained at approximately this level in the final samples - this change with time was significant ($p < 0.05$). Strep. salivarius, shown in Table 6.12., was undetected in most samples, under all treatment conditions, and at the two sampling times when it was isolated, it comprised less than 1 % of the total cultivable microflora.

Veillonella spp. comprised the total of the negative cocci isolated and, for the three treatment conditions combined, had its highest mean proportion in the 7-day samples, and the lowest at 21 days. This change in time was significant ($p < 0.05$). Similarly, the absolute counts rose between 2 and 7 days, before falling to an intermediate level in the final sample. The significance level for this change in count with time was 0.1 %. No significant treatment effect was seen for Veillonella spp., with regard to either proportional or absolute counts.

Positive bacilli showed a highly significant rise in proportion and absolute count with time in all treatment groups ($p < 0.001$), and a similar pattern was seen with Actinomyces spp., which was again significant at the 0.1 % level. No significant treatment effect occurred for either the group or the genus. At 7 days, where Actinomyces spp. comprised 21.2 - 37.7 % of the total flora, the most abundant species of this genus was A. odontolyticus which accounted for more than 50 % of the Actinomyces spp. under each treatment condition. However, at 21 days, the catalase negative A. viscosus / naeslundii (ie. A. naeslundii-like) organisms comprised the majority of Actinomyces spp.in the NP, SP and SPM plaques.

Lactobacillus spp. comprised a very low proportion of the total microflora in all plaque samples, and showed no pattern with time or treatment conditions. Nevertheless, the absolute counts rose with time in all treatment groups, the count at 2 days ranging from 0.04 - 0.47 $\log_{10}$ cfu per mm^2 enamel surface, while the range at 21 days was 1.53 - 2.60. This pattern was significant at the 1 % level.

Negative bacilli showed no significant change in proportion or count with time, and the mean proportion never comprised more than 6 % of the plaque microflora, at any time, in any treatment group. Haemophilus spp. had highest mean proportion in each treatment group in the initial sample, and indeed was not detected in any subsequent sample in either SP or SPM plaques. This fall with time was significant at the 5 % level and the slightly higher proportions found under normal plaque conditions was also significant at this level. Because of the low isolation frequency of this organism, absolute counts have not been given (see Section 6.2). The mean proportion of Bacteroides spp., under each treatment condition, rose from the 2-day value (significant at the 5 % level), while the absolute

counts also showed a significant time effect ($p < 0.01$), with the numbers rising from 2 - 7 days, and remaining at this level in the 21-day samples. With regard to both proportions and counts, no significant treatment effect was seen.

A histogram summarising the percentage distribution of Gram positive and negative cocci and bacilli, under each treatment condition, at each sampling time, is shown in Fig. 6.7.

6.3.3. Change in Microbial Composition of Plaque from Two Hours to Twenty One Days, in One Subject.

The 2-day and 21-day experimental runs were performed at different times, and 4 samples were obtained at each sampling time for each treatment condition in the 2 - 48 hour experiment, while six samples were obtained for each category in the three-week experiment. Thus, when combining results of the two experiments, four samples were available for each sampling time up to 30 hours, ten samples for 48 hours and six for 7 and 21 days. The proportional and absolute counts of the predominant microflora isolated in these experimental runs are given in Tables 6.1.and 6.3. (2-day experiment), and 6.11. and 6.13. (21-day experiment).

The total microbial count under NP conditions was similar at 2 and 6 hours, with values of 1.82 $\log_{10}$ cfu per mm^2 enamel surface and 1.88, respectively, before reaching a plateau at 48 hours, where the mean counts were 6.58 (in the 2-day experiment) and 6.84 (in the 21-day experiment). The counts remained static thereafter. In sucrose plaque samples, the maximum count was reached at 30 hours, with a value of approximately 7.00 $\log_{10}$ per mm^2 enamel surface, while under SPM conditions, the plateau count was reached at 48 hours, when the count was 6.78. In each case, the total count achieved was similar, although with the sucrose plaque samples the maximum count was attained more quickly.

The mean proportion of positive cocci, under all treatment conditions, rose slightly from the initial value (approximately 70 %) until 30 - 48 hours (approximately 80 %), after which it fell markedly to around 45 % in 7-day, and 33 % in 21-day samples.

Negative cocci showed no trend in mean proportion from 2 hours to 21 days, but were generally at their highest in 7-day samples. Positive bacilli fell from the values obtained at 2 hours (approximately 15 %) to a much lower level in samples taken at 10 - 30 hours. A small rise was seen at 48 hours, but a greater increase occurred thereafter, with this group forming approximately 30 % of the 7-day, and 50 % of the 21-day samples.

Negative bacilli accounted for approximately 10 % of the total microflora up to 24 hours, but generally fell subsequently, and at 21 days comprised around 4 % of the total organisms isolated.

Strep. mutans was either undetectable or present at 0.1 %, or less, of the total flora in the normal and sucrose plaques at all sampling times up to 21 days. In the SPM plaque, Strep. mutans fell from initially high levels (11.0 % at 2 hours and 28.1 % at 6 hours) to virtually undetectable levels up to 2 days. The organism constituted 13.1 % of the flora at 7 days and 18.0 % at 21 days. However, reference to absolute counts demonstrated a rise from 1.10 $\log_{10}$ cfu per mm^2 enamel surface at 2 hours to 5.60 at 7 days, although the counts at 10 and 24 hours did not fit the general trend. The count at 21 days was virtually unchanged from the 7-day value.

Table 6.1. Mean (SD) predominant cultivable plaque flora expressed as a percentage of the total flora, at 2 - 48 h, for Subject A, under each treatment condition, n=4.

SUBJECT A
NORMAL PLAQUE

	2h	6h	10h	24h	30h	48h
+ve cocci	72.7 (10.3)	80.8 (14.6)	88.9 (15.7)	83.8 (7.7)	92.2 (9.7)	74.7 (19.2)
S. mutans	ND	ND	ND	ND	ND	ND
S. sanguis	46.4 (0.4)	5.8 (11.6)	ND	14.2 (16.7)	2.6 (5.2)	41.3 (25.2)
S. oralis	26.2 (10.0)	75.0 (24.2)	88.9 (15.7)	69.6 (23.8)	89.6 (8.2)	33.4 (19.8)
-ve cocci	1.9 (2.7)	1.7 (3.4)	ND	ND	ND	9.1 (10.8)
Veillonella	1.9 (2.7)	1.7 (3.4)	ND	ND	ND	9.1 (10.8)
+ve bacilli	23.4 (4.9)	4.8 (3.9)	ND	8.3 (11.8)	ND	4.0 (4.6)
Actinomyces	21.6 (2.2)	1.9 (3.8)	ND	8.3 (11.8)	ND	2.1 (4.2)
Lactobacillus	ND	ND	ND	ND	ND	ND
-ve bacilli	1.9 (2.7)	12.6 (11.7)	11.1 (15.7)	7.9 (9.7)	7.8 (9.7)	12.2 (15.8)
Haemophilus	ND	ND	ND	ND	ND	ND
Bacteroides	1.9 (2.7)	11.8 (12.4)	11.1 (15.7)	7.9 (9.7)	7.8 (9.7)	12.2 (15.8)

SUBJECT A
SUCROSE PLAQUE

	2h	6h	10h	24h	30h	48h
+ve cocci	63.2 (28.4)	80.1 (23.9)	83.9 (13.2)	83.4 (13.9)	91.8 (7.0)	78.0 (35.5)
S. mutans	ND	ND	ND	ND	ND	ND
S. sanguis	13.0 (15.4)	ND	ND	ND	4.7 (9.4)	13.1 (15.1)
S. oralis	46.4 (17.6)	77.0 (22.8)	83.0 (14.0)	81.8 (13.7)	87.1 (10.6)	52.2 (48.6)
-ve cocci	6.6 (5.9)	7.9 (13.1)	2.1 (4.2)	2.8 (3.3)	2.4 (3.2)	7.5 (12.2)
Veillonella	6.6 (5.9)	2.2 (2.5)	ND	2.8 (3.3)	2.4 (3.2)	7.5 (12.2)
+ve bacilli	20.0 (14.5)	4.4 (6.4)	1.0 (2.1)	4.2 (2.9)	2.5 (5.0)	12.2 (24.4)
Actinomyces	18.6 (16.4)	4.4 (6.4)	1.0 (2.1)	4.2 (2.9)	2.5 (5.0)	12.2 (24.4)
Lactobacillus	ND	ND	ND	ND	ND	ND
-ve bacilli	10.2 (9.5)	6.5 (5.8)	13.0 (10.2)	9.4 (13.1)	3.3 (4.7)	2.3 (4.6)
Haemophilus	5.4 (10.7)	2.1 (4.2)	10.2 (12.2)	9.4 (13.1)	3.3 (4.7)	2.3 (4.6)
Bacteroides	1.2 (2.5)	3.3 (4.3)	2.8 (5.6)	ND	ND	ND

Table 6.1. cont.

SUBJECT A
SPM

	2h	6h	10h	24h	30h	48h
+ve cocci	76.5 (22.1)	81.9 (16.0)	64.5 (25.9)	89.7 (9.9)	93.9 (1.6)	90.6 (4.2)
S. mutans	11.0 (7.4)	28.1 (48.0)	ND	0.1 (0.0)	0.1 (0.0)	0.1 (0.0)
S. sanguis	1.9 (3.8)	ND	14.2 (21.9)	8.2 (7.7)	ND	4.0 (2.8)
S. oralis	65.7 (18.3)	43.5 (38.5)	48.2 (30.2)	78.6 (12.6)	93.9 (1.6)	84.9 (4.4)
-ve cocci	12.6 (12.8)	7.0 (10.2)	23.8 (27.0)	0.8 (1.7)	3.7 (4.3)	4.4 (6.3)
Veillonella	10.8 (11.4)	6.4 (10.7)	8.5 (12.4)	0.8 (1.7)	3.7 (4.3)	4.4 (6.3)
+ve bacilli	1.8 (2.1)	0.8 (1.6)	5.9 (7.5)	3.1 (6.2)	ND	2.9 (3.4)
Actinomyces	1.8 (2.1)	0.8 (1.6)	5.9 (7.5)	3.1 (6.2)	ND	2.9 (3.4)
Lactobacillus	ND	ND	ND	ND	ND	ND
-ve bacilli	9.0 (11.9)	6.1 (7.2)	6.0 (1.4)	6.3 (4.2)	2.3 (2.7)	2.1 (4.2)
Haemophilus	0.9 (1.8)	3.0 (6.0)	2.5 (2.9)	4.0 (5.9)	1.1 (2.2)	2.1 (4.2)
Bacteroides	6.2 (12.5)	1.7 (3.4)	3.5 (4.1)	2.4 (2.8)	1.2 (2.4)	ND

Table 6.1. cont.

Table 6.2. Mean (SD) microbial counts (log_{10} cfu per mm^2 enamel slab surface) of predominant plaque microflora, at 2 - 48 h, for Subject A, under each treatment condition, n=4.

SUBJECT A
NORMAL PLAQUE

	2h	6h	10h	24h	30h	48h
+ve cocci	1.64 (1.72)	1.81 (0.73)	2.64 (0.29)	4.24 (1.43)	5.52 (0.85)	6.50 (0.24)
S. mutans	0.33 (0.08)	0.27 (0.19)	0.30 (0.08)	0.31 (0.21)	0.33 (0.09)	0.33 (0.03)
S. sanguis	1.54 (1.61)	0.90 (0.91)	1.39 (1.04)	3.06 (1.08)	3.81 (0.88)	6.16 (0.15)
S. oralis	1.42 (1.52)	1.76 (0.69)	2.64 (0.29)	4.14 (1.49)	5.50 (0.86)	6.10 (0.52)
-ve cocci	0.88 (0.80)	0.60 (0.71)	1.39 (1.04)	2.50 (1.02)	3.54 (0.93)	5.30 (0.71)
Veillonella	0.88 (0.80)	0.60 (0.71)	1.39 (1.04)	2.50 (1.02)	3.54 (0.93)	5.30 (0.71)
+ve bacilli	1.40 (1.44)	0.99 (0.66)	1.39 (1.04)	2.91 (0.93)	3.54 (0.93)	5.16 (0.81)
Actinomyces	1.38 (1.43)	0.79 (0.76)	1.39 (1.04)	2.91 (0.93)	3.54 (0.93)	4.97 (0.76)
-ve bacilli	0.88 (0.80)	1.20 (0.81)	1.56 (1.28)	3.11 (1.53)	4.13 (1.61)	5.36 (1.07)
Bacteroides	0.88 (0.80)	1.18 (0.80)	1.56 (1.28)	3.11 (1.53)	4.13 (1.61)	5.36 (1.07)
Total	1.82 (1.64)	1.88 (0.78)	2.65 (0.35)	4.25 (1.38)	5.52 (0.92)	6.58 (0.31)

SUBJECT A
SUCROSE PLAQUE

	2h	6h	10h	24h	30h	48h
+ve cocci	2.14 (0.98)	2.71 (0.88)	3.64 (1.26)	5.63 (0.82)	7.02 (0.82)	6.71 (1.09)
S. mutans	0.32 (0.03)	0.42 (0.03)	0.36 (0.04)	0.44 (0.08)	0.35 (0.07)	0.38 (0.08)
S. sanguis	1.34 (1.28)	1.18 (0.56)	1.74 (1.28)	3.83 (0.78)	5.43 (1.15)	5.63 (1.32)
S. oralis	2.02 (0.82)	2.70 (0.88)	3.63 (1.26)	5.62 (0.83)	6.99 (0.78)	6.06 (1.02)
-ve cocci	1.10 (0.86)	1.63 (0.52)	1.97 (1.00)	4.17 (0.69)	5.50 (1.09)	5.35 (1.66)
Veillonella	1.10 (0.86)	1.47 (0.65)	1.74 (1.28)	4.17 (0.69)	5.50 (1.09)	5.35 (1.66)
+ve bacilli	1.59 (0.70)	1.57 (0.38)	1.91 (1.06)	4.36 (0.79)	5.33 (0.72)	5.25 (1.68)
Actinomyces	1.59 (0.70)	1.57 (0.38)	1.91 (1.06)	4.36 (0.79)	5.33 (0.72)	5.25 (1.68)
-ve bacilli	1.21 (0.88)	1.81 (0.67)	2.65 (1.57)	4.41 (1.33)	5.54 (1.14)	5.07 (1.34)
Bacteroides	0.81 (1.00)	1.53 (0.42)	1.97 (1.67)	3.83 (0.78)	5.09 (0.63)	4.83 (1.04)
Total	2.32 (0.85)	2.78 (0.79)	3.68 (1.24)	5.68 (0.85)	7.00 (0.84)	6.68 (1.07)

Table 6.2. cont.

SUBJECT A
SPM

	2h	6h	10h	24h	30h	48h
+ve cocci	1.89 (0.63)	2.95 (1.27)	2.85 (1.58)	5.11 (0.76)	6.01 (2.02)	6.80 (0.79)
S. mutans	1.10 (0.69)	2.06 (1.87)	0.29 (0.17)	1.41 (0.80)	2.41 (0.49)	2.73 (0.28)
S. sanguis	0.49 (0.42)	1.14 (1.32)	1.82 (1.13)	3.90 (1.02)	4.04 (1.99)	5.46 (0.91)
S. oralis	1.82 (0.59)	2.38 (0.48)	2.72 (1.70)	5.07 (0.74)	6.01 (2.02)	6.78 (0.81)
-ve cocci	0.95 (0.44)	1.73 (1.05)	2.20 (1.05)	3.38 (0.88)	4.52 (2.22)	5.38 (0.95)
Veillonella	0.91 (0.40)	1.61 (1.21)	1.78 (1.23)	3.38 (0.88)	4.52 (2.22)	5.10 (1.01)
+ve bacilli	0.79 (0.42)	1.31 (1.22)	1.72 (1.22)	3.46 (1.31)	4.04 (1.99)	5.28 (0.68)
Actinomyces	0.61 (0.53)	1.31 (1.22)	1.72 (1.22)	3.46 (1.31)	4.04 (1.99)	5.28 (0.68)
-ve bacilli	0.50 (0.26)	1.69 (0.97)	1.96 (1.45)	3.98 (0.97)	4.40 (1.91)	5.17 (1.13)
Bacteroides	0.50 (0.26)	1.38 (1.20)	1.61 (1.66)	3.38 (0.74)	4.21 (1.68)	4.92 (0.70)
Total	1.95 (0.53)	2.98 (1.23)	3.02 (1.51)	5.12 (0.78)	6.00 (2.02)	6.78 (0.79)

Table 6.2. cont.

SUBJECT A
PERCENTAGE COUNT

	Time	Treatment	Position	Side
+ve cocci	NS	NS	NS	NS
S. mutans	NS	NS	NS	NS
S. sanguis	**	**	NS	NS
S. oralis	**	NS	NS	NS
-ve cocci	NS	NS	NS	NS
Veillonella	NS	NS	NS	NS
+ve bacilli	*	NS	NS	NS
Actinomyces	*	NS	NS	*
Lactobacillus	NS	NS	NS	NS
-ve bacilli	NS	NS	NS	NS
Haemophilus	NS	**	NS	NS
Bacteroides	NS	***	NS	NS

NS = Not significant ; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Table 6.3. Statistical analysis of effect of time, treatment, position and side, on percentage predominant plaque microflora isolated from Subject A, at 2 - 48 h.

SUBJECT A
ABSOLUTE COUNT

	Time	Treatment	Position	Side
+ve cocci	***	*	NS	NS
S. mutans	*	***	NS	NS
S. sanguis	***	*	NS	NS
S. oralis	***	*	NS	NS
-ve cocci	***	*	NS	NS
Veillonella	***	*	NS	NS
+ve cocci	***	NS	NS	NS
Actinomyces	***	*	NS	NS
-ve bacillus	***	NS	NS	NS
Bacteroides	***	NS	NS	NS
Total	***	*	NS	NS

NS = Not significant ; * = $p < 0.05$; *** = $p < 0.001$.

Table 6.4. Statistical analysis of effect of time, treatment, position and side, on microbial counts of predominant plaque microflora isolated from Subject A, at 2 - 48 h.

SUBJECT A
ACTINOMYCES SPECIES

NORMAL PLAQUE

	2h	6h	10h	24h	30h	48h
A. odontolyticus	ND	ND	ND	6.2 (12.5)	ND	ND
A. v/n cat. +ve	6.6 (9.4)	ND	ND	ND	ND	ND
A. v/n cat. -ve	14.9 (11.6)	1.9 (3.8)	ND	2.1 (4.2)	ND	2.1 (4.2)

SUCROSE PLAQUE

	2h	6h	10h	24h	30h	48h
A. odontolyticus	3.6 (7.2)	2.3 (4.6)	1.0 (2.1)	4.2 (2.9)	2.5 (5.0)	12.2 (24.4)
A. v/n cat. +ve	6.1 (7.2)	2.2 (2.5)	ND	ND	ND	ND
A. v/n cat. -ve	8.9 (13.5)	ND	ND	ND	ND	ND

SPM

	2h	6h	10h	24h	30h	48h
A. odontolyticus	0.8 (1.7)	ND	4.2 (6.4)	3.1 (6.2)	ND	2.9 (3.4)
A. v/n cat. +ve	ND	ND	1.6 (2.7)	ND	ND	ND
A. v/n cat. -ve	1.0 (1.9)	0.8 (1.6)	ND	ND	ND	ND

Table 6.5. Mean (SD) percentage distribution of A. odontolyticus and catalase positive and negative divisions of A. viscosus/naeslundii, at 2 - 48 h for Subject A, under each treatment condition, n=4.

Table 6.6. Mean (SD) predominant cultivable plaque flora expressed as a percentage of the total microflora, at 2 - 48 h, for three subjects, under NP and SP conditions, n=8.

ALL SUBJECTS
NORMAL PLAQUE

	2h	6h	10h	24h	30h	48h
+ve cocci	76.9 (17.2)	79.8 (18.7)	83.2 (15.6)	80.9 (9.4)	82.0 (14.9)	71.6 (17.0)
S. mutans	0.3 (0.7)	0.2 (0.4)	0.03 (0.1)	0.2 (0.4)	0.01 (0.04)	0.05 (0.05)
S. sanguis	20.5 (23.2)	11.0 (12.8)	11.2 (11.8)	19.5 (17.1)	8.9 (11.9)	35.6 (19.0)
S. oralis	41.9 (17.8)	65.7 (23.7)	68.5 (22.1)	59.4 (23.2)	70.2 (23.8)	35.0 (17.2)
-ve cocci	3.3 (4.2)	4.7 (7.1)	7.2 (10.1)	5.0 (7.5)	2.4 (3.5)	10.7 (9.0)
Veillonella	2.3 (4.1)	4.7 (7.1)	6.0 (8.0)	0.5 (1.5)	0.5 (1.5)	5.3 (8.4)
+ve bacilli	10.7 (11.1)	8.1 (10.1)	ND	7.5 (9.8)	4.4 (9.5)	8.4 (9.1)
Actinomyces	9.0 (10.0)	6.6 (10.7)	ND	7.2 (10.0)	3.9 (9.3)	7.2 (8.9)
Lactobacillus	ND	ND	ND	0.3 (0.8)	0.5 (1.5)	0.4 (1.1)
-ve bacilli	9.0 (20.2)	7.4 (9.9)	9.6 (9.0)	6.6 (8.3)	10.0 (9.8)	8.6 (11.6)
Haemophilus	8.3 (20.4)	ND	5.9 (7.1)	2.6 (5.9)	6.1 (9.7)	2.5 (4.4)
Bacteroides	0.6 (1.6)	6.9 (10.0)	3.7 (9.1)	3.9 (7.6)	3.9 (7.6)	6.1 (12.2)

ALL SUBJECTS
SUCROSE PLAQUE

	2h	6h	10h	24h	30h	48h
+ve cocci	71.6 (22.4)	86.0 (17.8)	83.0 (11.6)	71.7 (20.0)	88.2 (8.4)	66.5 (27.5)
S. mutans	2.1 (5.0)	ND	0.2 (0.4)	0.7 (1.3)	0.05 (0.05)	0.2 (0.3)
S. sanguis	12.9 (16.7)	8.8 (17.0)	5.3 (10.3)	2.3 (3.9)	7.6 (9.7)	16.8 (27.2)
S. oralis	45.8 (18.2)	67.7 (20.3)	72.0 (22.2)	68.6 (20.0)	78.9 (12.8)	48.7 (32.3)
-ve cocci	5.4 (6.8)	3.9 (9.6)	5.8 (9.4)	13.7 (17.9)	2.7 (3.3)	14.0 (11.8)
Veillonella	5.4 (6.8)	1.1 (2.0)	3.2 (6.6)	3.5 (4.6)	2.0 (2.5)	12.7 (11.6)
+ve bacilli	13.5 (13.9)	5.2 (7.6)	2.8 (5.3)	8.1 (9.2)	4.0 (6.8)	15.7 (18.3)
Actinomyces	12.8 (14.5)	5.2 (7.6)	1.8 (2.9)	7.6 (8.7)	4.0 (6.8)	14.6 (18.4)
Lactobacillus	ND	ND	ND	ND	ND	1.1 (3.1)
-ve bacilli	9.0 (8.7)	4.3 (5.2)	8.4 (8.9)	6.5 (9.5)	5.1 (6.6)	2.6 (4.0)
Haemophilus	2.7 (7.6)	2.1 (3.8)	6.7 (9.2)	6.5 (9.5)	2.4 (3.4)	2.6 (4.0)
Bacteroides	2.7 (5.9)	1.7 (3.3)	1.7 (3.9)	ND	2.7 (5.2)	ND

Table 6.6. cont.

Table 6.7. Total cultivable plaque flora, expressed as percentage of the total microflora, at 2 - 48 h, for three subjects combined, under NP and SP conditions, n=8.

ALL SUBJECTS
NORMAL PLAQUE

	2h	6h	10h	24h	30h	48h
+ve cocci	76.9	79.8	83.2	80.9	82.0	71.6
S. mutans	0.3	0.2	0.03	0.2	0.01	0.05
S. sanguis	20.5	11.0	11.2	19.5	8.9	35.6
S. oralis	41.9	65.7	68.5	59.4	70.2	35.0
S. salivarius	5.2	1.0	2.1	ND	1.8	0.9
S. milleri	1.0	2.1	ND	0.4	ND	ND
Other Strep.	ND	ND	ND	1.2	ND	ND
ANO_2 Strep.	ND	ND	0.5	ND	0.5	ND
Micrococcus/Staph	8.3	ND	ND	ND	0.5	ND
-ve cocci	3.3	4.7	7.2	5.0	2.4	10.7
Veillonella	2.3	4.7	6.0	0.5	0.5	5.3
Neisseria	1.0	ND	1.1	4.5	1.9	5.4
+ve bacilli	10.7	8.1	ND	7.5	4.4	8.4
A.odontolyticus	1.9	3.6	ND	6.2	3.9	6.2
A. v/n cat. +ve	2.2	ND	ND	ND	ND	ND
A. v/n cat. -ve	5.0	3.0	ND	1.0	ND	1.0
Lactobacillus	ND	ND	ND	0.3	0.5	0.4
Propionibacterium	0.8	ND	ND	ND	ND	ND
Unidentified	0.5	1.5	ND	ND	ND	1.0
-ve bacilli	9.0	7.4	9.6	6.6	10.0	8.6
Haemophilus	8.3	ND	5.9	2.6	6.1	2.5
Bacteroides	0.6	6.9	3.7	3.9	3.9	6.1
Unidentified	ND	ND	ND	ND	1.5	0.6

ALL SUBJECTS
SUCROSE PLAQUE

	2h	6h	10h	24h	30h	48h
+ve cocci	71.6	86.0	83.0	71.7	88.2	66.5
S. mutans	2.1	ND	0.2	0.7	0.05	0.2
S. sanguis	12.9	8.8	5.3	2.3	7.6	16.8
S. oralis	45.8	67.7	72.0	68.6	78.9	48.7
S. salivarius	10.5	5.6	4.9	ND	1.6	0.7
S. milleri	0.6	0.5	0.4	ND	ND	ND
ANO_2 Strep.	ND	1.0	ND	ND	ND	0.3
Micrococcus/Staph.	ND	1.3	0.4	ND	ND	ND
-ve cocci	5.4	3.9	5.8	13.7	2.7	14.0
Veillonella	5.4	1.1	3.2	3.5	2.0	12.7
Neisseria	ND	2.8	2.5	10.2	0.7	1.2
+ve bacilli	13.5	5.2	2.8	8.1	4.0	15.7
A. odontolyticus	5.3	1.6	1.8	7.6	4.0	13.5
A. v/n cat. +ve	3.0	1.1	ND	ND	ND	1.1
A. v/n cat. -ve	4.5	2.5	ND	ND	ND	ND
Lactobacillus	ND	ND	ND	ND	ND	1.1
Propionibacterium	ND	ND	1.0	ND	ND	ND
Unidentified	0.7	ND	ND	0.4	ND	ND
-ve bacilli	9.0	4.3	8.4	6.5	5.1	2.6
Haemophilus	2.7	2.1	6.7	6.5	2.4	2.6
Eikenella	1.8	ND	ND	ND	ND	ND
Bacteroides	2.7	1.7	1.7	ND	2.7	ND
Fusobacterium	1.8	0.6	ND	ND	ND	ND
Unidentified	0.6	ND	ND	ND	ND	1.1

Table 6.7. cont.

Table 6.8. Mean (SD) microbial counts (log_{10} cfu per mm^2 enamel slab surface) of predominant plaque microflora at 2 - 48 h, for three subjects combined, under NP and SP conditions, n=8.

ALL SUBJECTS
NORMAL PLAQUE

	2h	6h	10h	24h	30h	48h
+ve cocci	1.65 (1.28)	2.03 (0.74)	3.29 (1.71)	4.48 (1.28)	5.66 (0.72)	6.33 (0.57)
S. mutans	0.27 (0.14)	0.45 (0.38)	0.70 (0.72)	0.99 (0.95)	0.61 (0.56	1.42 (1.27)
S. sanguis	1.12 (1.37)	1.18 (0.94)	2.40 (1.94)	3.60 (1.10)	4.38 (1.13)	5.94 (0.64)
S. oralis	1.44 (1.12)	0.71 (0.82)	2.15 (1.41)	2.85 (1.06)	3.86 (0.91)	4.98 (0.78)
-ve cocci	0.83 (0.78)	0.82 (0.53)	2.18 (1.40)	3.14 (1.22)	4.07 (1.08)	5.35 (0.79)
Veillonella	0.74 (0.82)	0.82 (0.53)	2.15 (1.41)	2.85 (1.06)	3.86 (0.91)	4.98 (0.78)
+ve bacilli	1.08 (1.12)	1.04 (0.45)	1.72 (1.52)	3.22 (1.28)	4.05 (1.05)	5.27 (0.63)
Actinomyces	1.04 (1.12)	0.94 (0.53)	1.72 (1.52)	3.16 (1.31)	4.02 (1.01)	5.16 (0.62)
-ve bacilli	0.71 (0.64)	1.00 (0.64)	2.19 (1.43)	3.30 (1.23)	4.49 (1.41)	5.19 (0.90)
Bacteroides	0.62 (0.67)	0.99 (0.63)	1.78 (1.55)	3.06 (1.35)	4.07 (1.14)	4.92 (0.97)
Total	1.76 (1.22)	2.11 (0.71)	3.33 (1.65)	4.52 (1.24)	5.72 (0.78)	6.44 (0.56)

ALL SUBJECTS
SUCROSE PLAQUE

	2h	6h	10h	24h	30h	48h
+ve cocci	2.15 (0.87)	2.40 (0.70)	3.90 (1.22)	5.07 (1.36)	6.59 (0.84)	6.52 (0.76)
S. mutans	0.54 (0.43)	0.30 (0.20)	0.89 (0.89)	1.66 (1.92)	1.12 (1.30)	1.69 (1.96)
S. sanguis	1.26 (1.19)	1.04 (0.74)	2.43 (1.36)	3.60 (1.06)	5.36 (0.91)	5.41 (0.99)
S. oralis	1.96 (0.82)	2.30 (0.76)	3.82 (1.27)	5.05 (1.38)	6.53 (0.83)	6.15 (0.70)
-ve cocci	0.96 (0.68)	0.91 (0.86)	2.51 (1.23)	4.02 (1.01)	5.21 (0.93)	5.62 (1.14)
Veillonella	0.96 (0.68)	0.83 (0.83)	2.31 (1.30)	3.74 (1.31)	5.16 (0.91)	5.59 (1.14)
+ve bacilli	1.34 (0.93)	1.16 (0.71)	2.37 (1.36)	4.03 (1.54)	5.11 (0.59)	5.53 (1.19)
Actinomyces	1.34 (0.93)	1.16 (0.71)	2.33 (1.32)	4.02 (1.52)	5.11 (0.59)	5.50 (1.17)
-ve bacilli	1.10 (0.62)	1.08 (0.94)	2.73 (1.18)	3.87 (1.54)	5.32 (1.01)	5.07 (0.96)
Bacteroides	0.81 (0.74)	0.86 (0.79)	2.28 (1.36)	3.37 (1.20)	5.06 (0.72)	4.78 (0.70)
Total	2.26 (0.80)	2.44 (0.67)	3.92 (1.20)	5.18 (1.36)	6.59 (0.84)	6.60 (0.73)

Table 6.8. cont.

ALL SUBJECTS
PERCENTAGE COUNT

	Time	Treatment	Position	Side	Subject
+ve cocci	*	NS	NS	*	*
S. mutans	NS	NS	NS	NS	NS
S. sanguis	*	*	NS	NS	NS
S. oralis	***	NS	NS	NS	***
-ve cocci	**	*	NS	NS	***
Veillonella	*	NS	NS	NS	NS
+ve bacilli	*	NS	NS	NS	NS
Actinomyces	*	*	NS	NS	NS
Lactobacillus	NS	NS	NS	NS	*
-ve bacilli	NS	NS	NS	NS	***
Haemophilus	NS	*	NS	*	***
Bacteroides	NS	**	NS	*	**

NS = Not significant ; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

Table 6.9. Statistical analysis of effect of time, treatment, position, side and subject on percentage predominant plaque microflora, isolated from three subjects, at 2 - 48 h.

ALL SUBJECTS
ABSOLUTE COUNTS

	Time	Treatment	Position	Side	Subject
+ve cocci	***	**	NS	NS	**
S. mutans	**	NS	NS	NS	***
S. sanguis	***	NS	NS	NS	*
S. oralis	***	**	NS	NS	**
-ve cocci	***	**	NS	*	NS
Veillonella	***	**	NS	NS	NS
+ve bacilli	***	**	NS	NS	***
Actinomyces	***	**	NS	NS	***
-ve bacilli	***	*	NS	NS	NS
Bacteroides	***	NS	NS	NS	*
Total	***	**	NS	NS	**

NS = Not significant ; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

Table 6.10. Statistical analysis of effect of time, treatment, position, side and subject on microbial counts of predominant plaque microflora, isolated from three subjects, at 2 - 48 h.

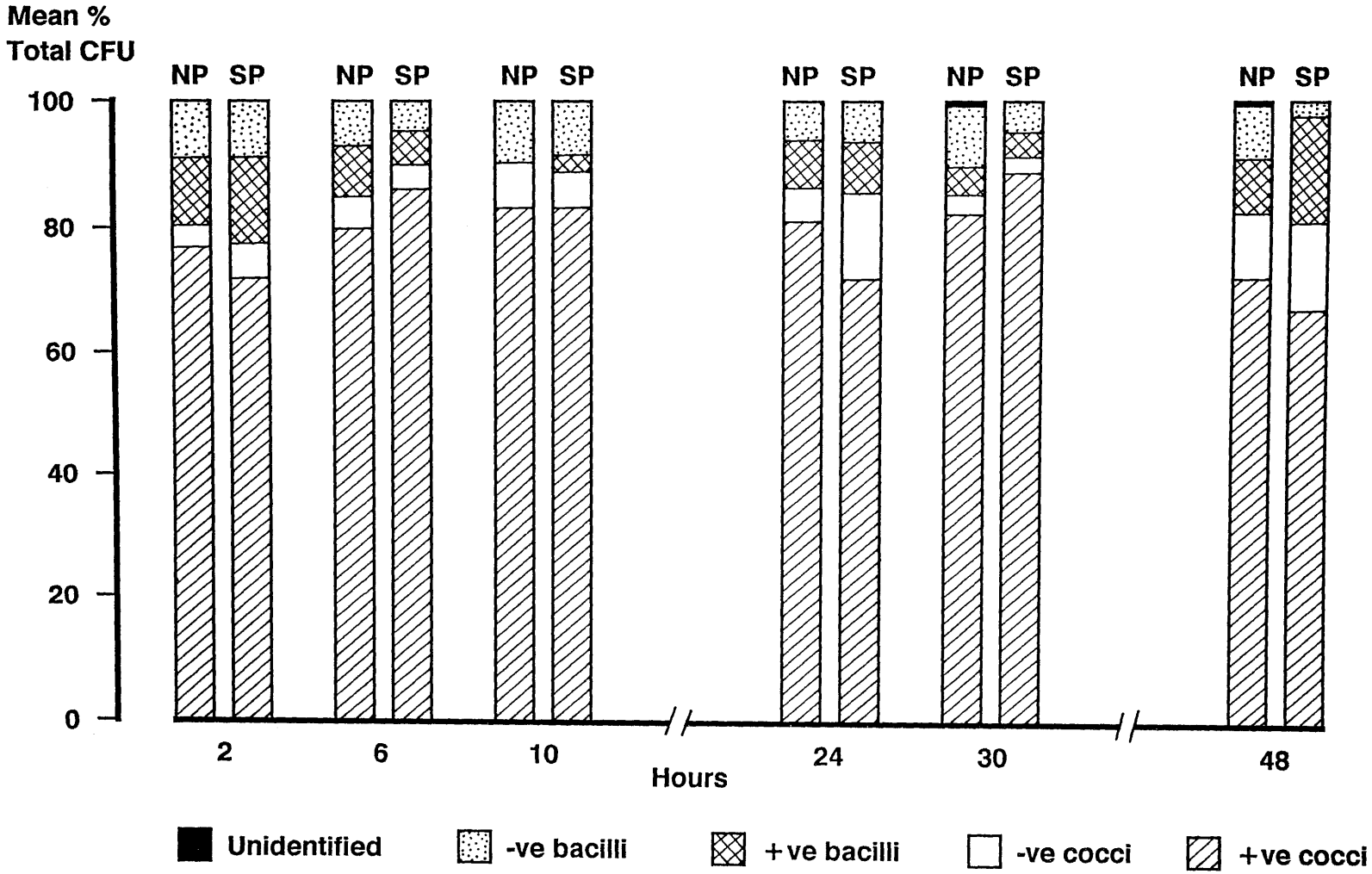


Fig. 6.2. Mean percentage distribution of Gram positive and negative cocci and bacilli, for all subjects, under NP and SP conditions, at 2 - 48 hours, n=8.

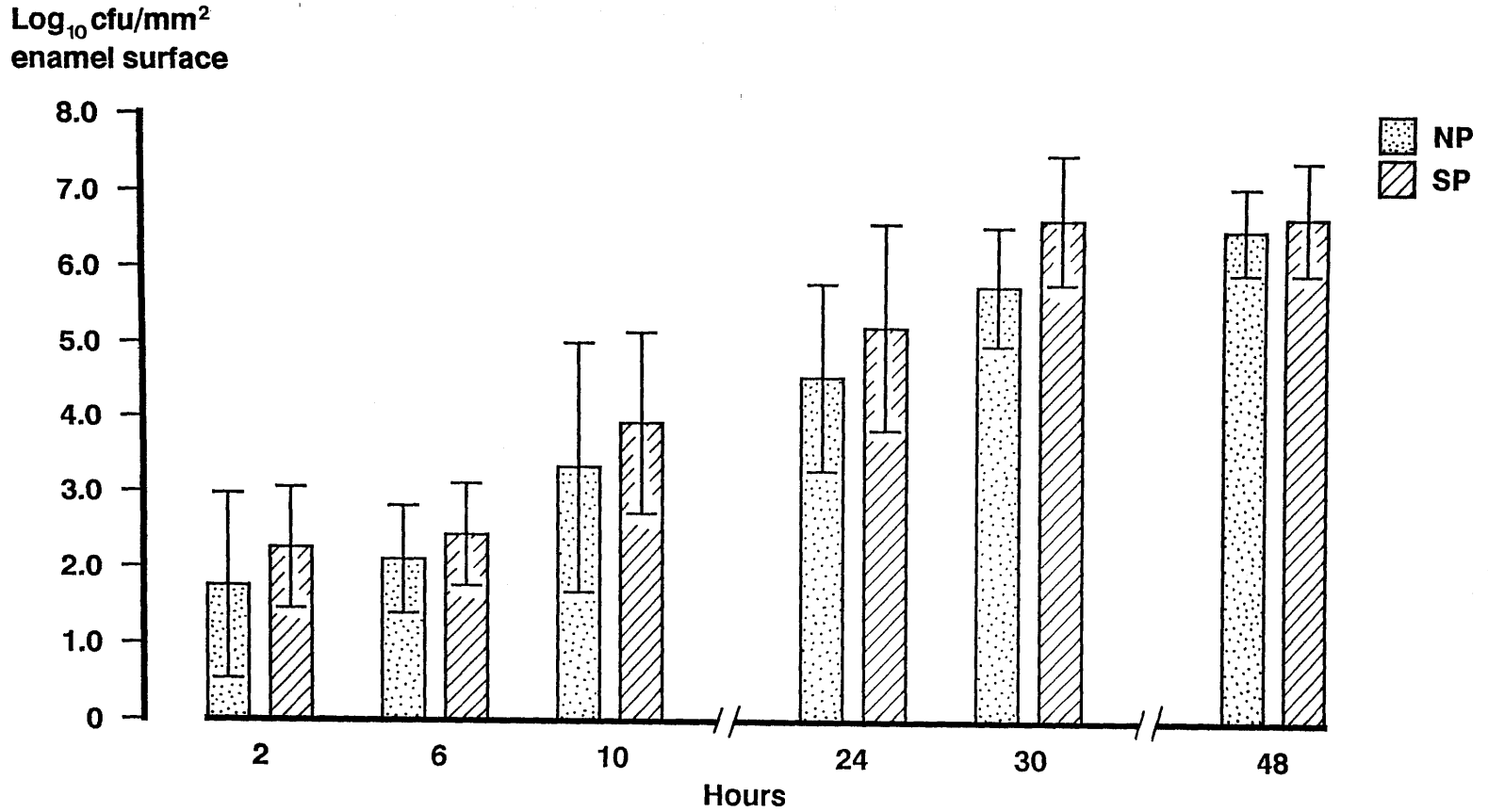


Fig. 6.3. Mean (SD) total counts (log_{10} cfu per mm^2 enamel surface) for all subjects, under NP and SP conditions, at 2 - 48 hours, n=8.

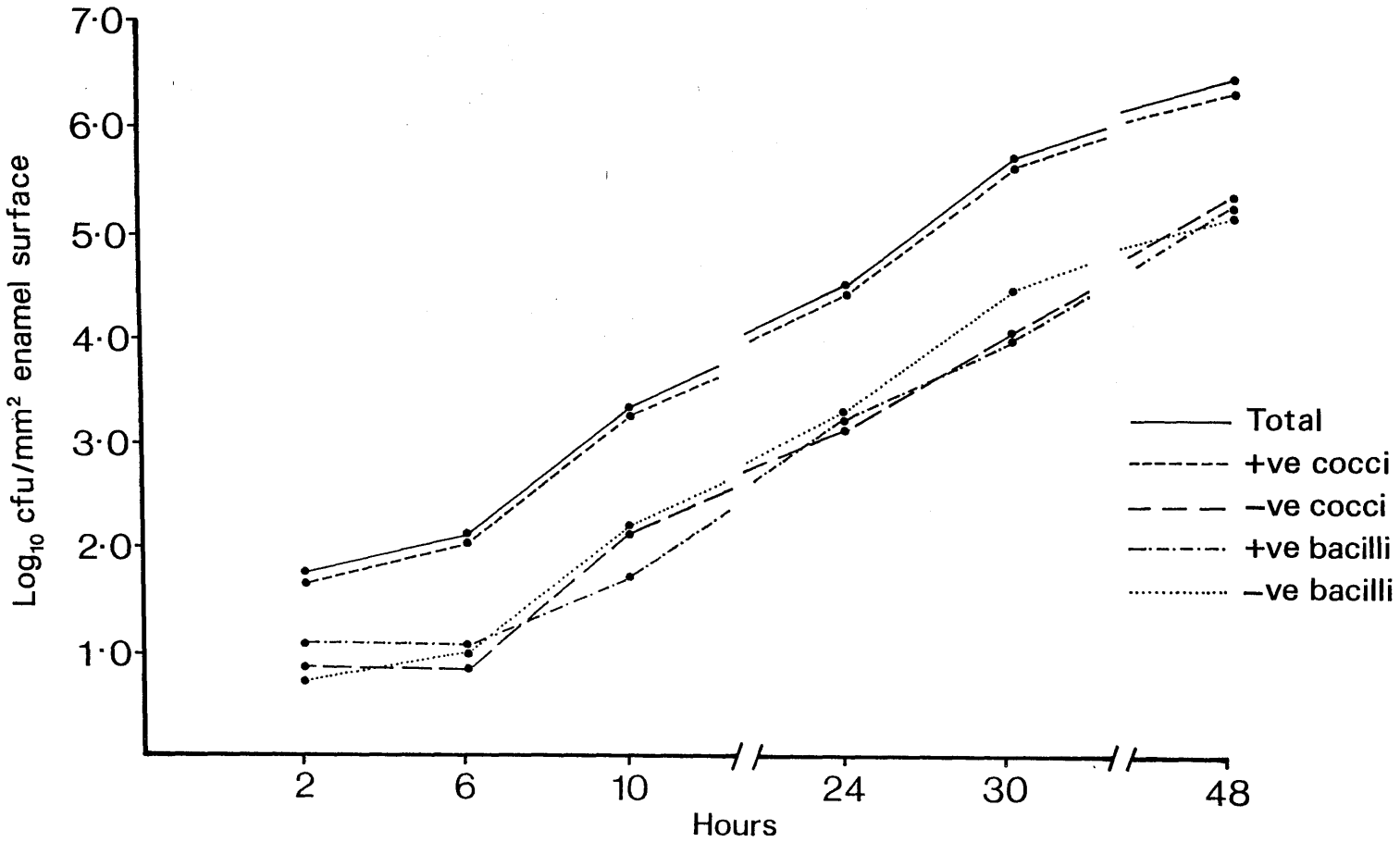


Fig. 6.4. Mean microbial counts (log_{10} cfu per mm^2 enamel surface) of Gram positive and negative cocci and bacilli, and total counts, for all subjects, under NP conditions, at 2 - 48 hours, n=8.

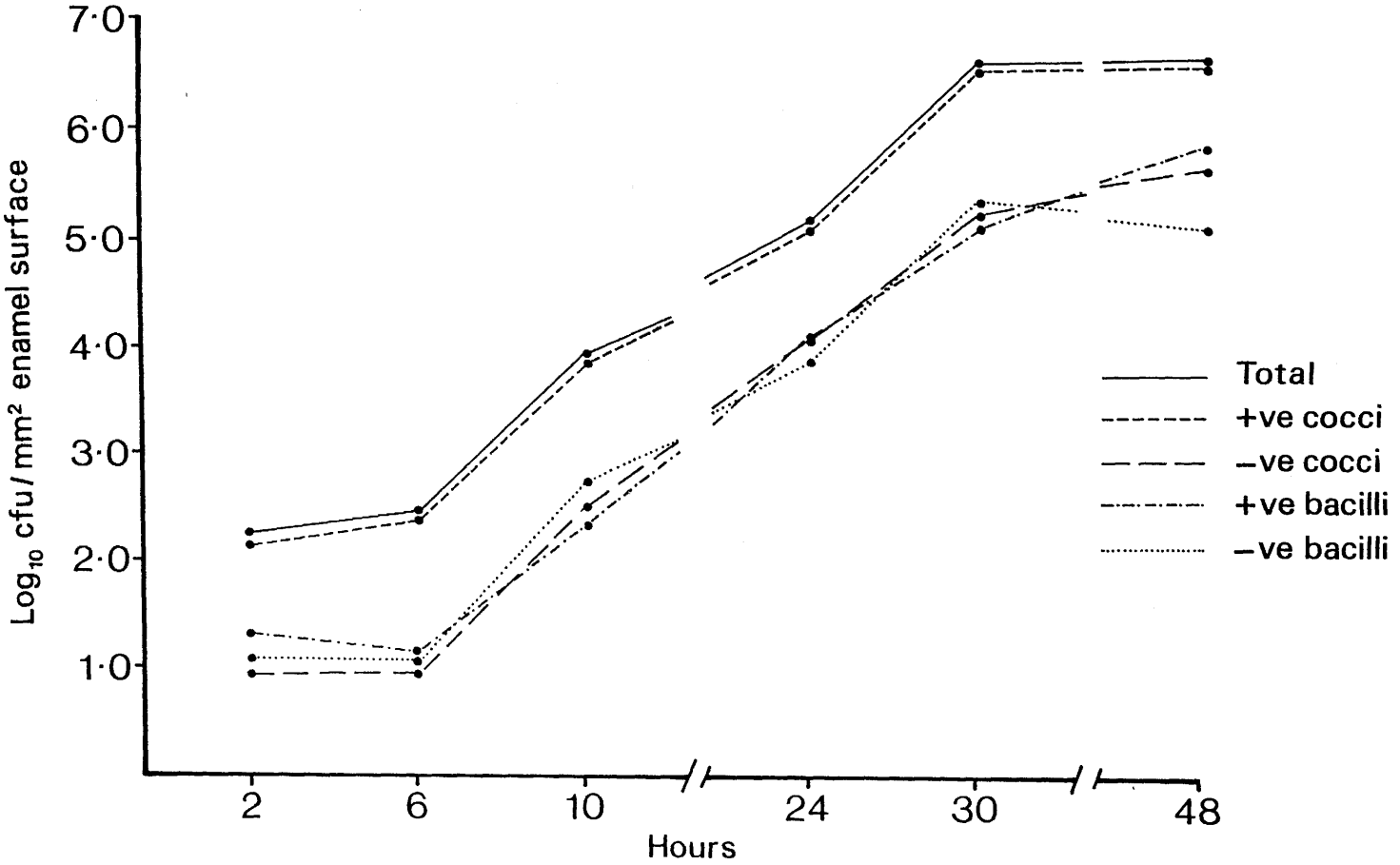


Fig. 6.5. Mean microbial counts (log_{10} cfu per mm^2 enamel surface) of Gram positive and negative cocci and bacilli, and total counts, for all subjects, under SP conditions, at 2 - 48 hours, n=8.

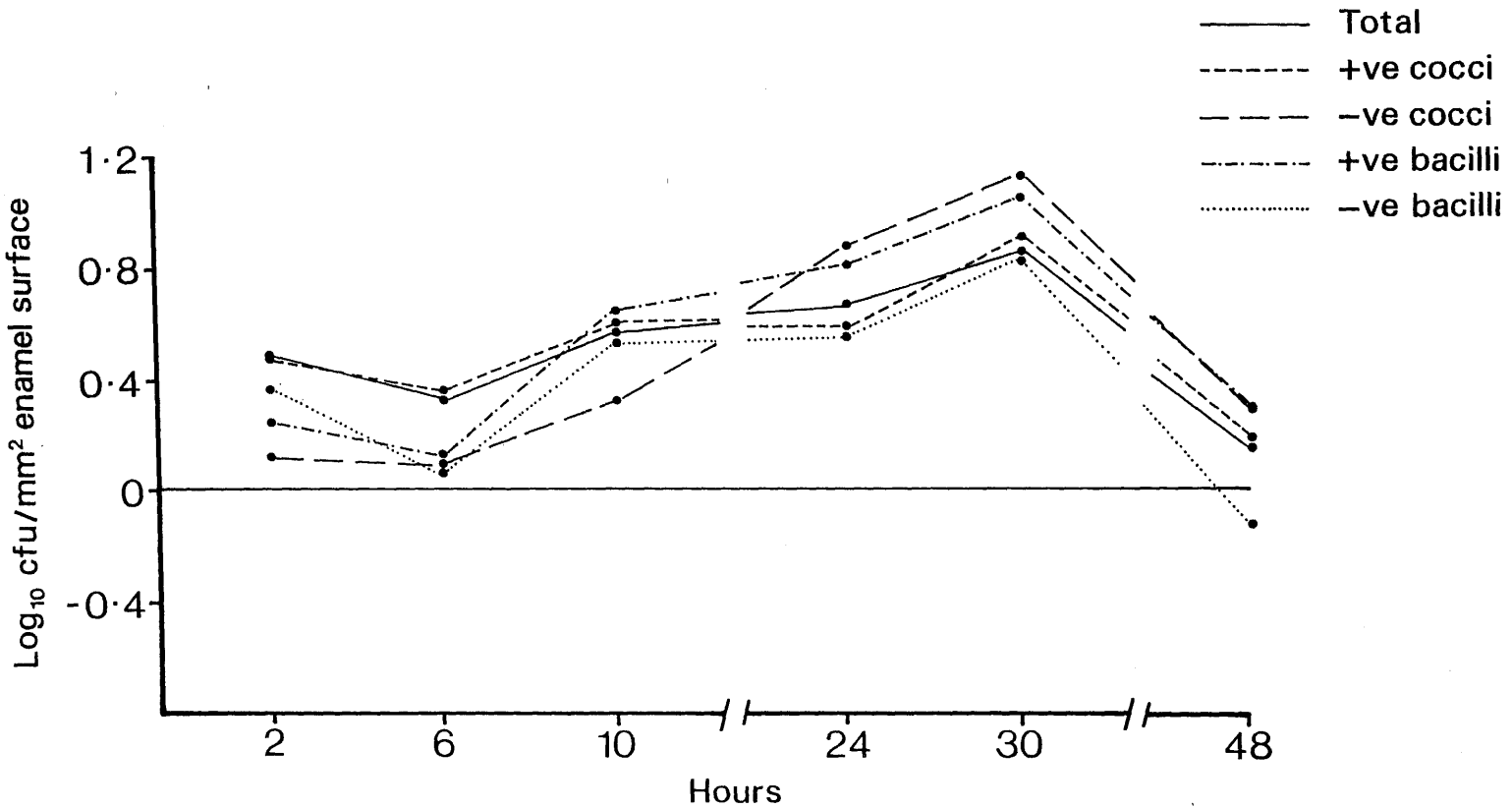


Fig. 6.6. Mean difference in microbial counts (log_{10} cfu per mm^2 enamel surface) of Gram positive and negative cocci and bacilli, and total counts, between SP and NP plaques, for all subjects, at 2 - 48 hours, n=8.

Table 6.11. Mean (SD) predominant plaque flora, expressed as percentage of total microflora, at 2, 7 and 21 days, from one subject, under each treatment condition, n=6.

NORMAL PLAQUE

	2 day	7 day	21 day
+ve cocci	92.4 (4.9)	32.4 (18.0)	25.6 (25.7)
S. mutans	0.03 (0.05)	0.03 (0.05)	0.04 (0.05)
S. sanguis	5.4 (9.3)	13.5 (11.2)	20.7 (27.6)
S. oralis	85.6 (9.9)	16.5 (14.1)	3.3 (4.1)
-ve cocci	1.3 (2.7)	28.2 (13.3)	12.0 (16.5)
Veillonella	1.3 (2.7)	28.2 (13.3)	12.0 (16.5)
+ve bacilli	2.2 (1.9)	37.7 (9.6)	55.5 (27.0)
Actinomyces	2.2 (1.9)	36.2 (8.8)	55.3 (26.8)
Lactobacillus	ND	0.02 (0.04)	0.3 (0.5)
-ve bacilli	4.1 (3.7)	1.7 (2.6)	6.0 (4.4)
Haemophilus	3.9 (3.8)	0.8 (2.0)	0.8 (1.8)
Bacteroides	0.2 (0.4)	0.8 (2.0)	1.7 (2.3)

SUCROSE PLAQUE

	2 day	7 day	21 day
+ve cocci	76.3 (17.8)	55.4 (13.0)	46.0 (19.2)
S. mutans	0.1 (0.04)	0.1 (0.05)	0.1 (0.04)
S. sanguis	33.9 (20.0)	42.4 (11.4)	18.6 (12.8)
S. oralis	41.6 (34.1)	9.4 (9.2)	18.6 (12.8)
-ve cocci	17.1 (19.3)	12.2 (4.4)	6.3 (2.8)
Veillonella	17.1 (19.3)	12.2 (4.4)	6.3 (2.8)
+ve bacilli	4.1 (3.4)	26.7 (11.0)	42.3 (19.1)
Actinomyces	4.1 (3.4)	26.1 (11.9)	37.5 (19.2)
Lactobacillus	0.02 (0.04)	0.02 (0.04)	1.6 (2.5)
-ve bacilli	2.5 (5.6)	5.7 (7.2)	5.4 (4.0)
Haemophilus	0.8 (1.9)	ND	ND
Bacteroides	ND	2.7 (2.9)	3.0 (3.7)

SPM

	2 day	7 day	21 day
+ve cocci	88.0 (9.0)	56.8 (25.5)	30.1 (16.7)
S. mutans	1.6 (3.0)	13.1 (16.1)	18.0 (20.7)
S. sanguis	21.4 (22.9)	27.4 (20.2)	6.2 (7.9)
S. oralis	62.7 (27.8)	16.0 (14.3)	5.6 (7.8)
-ve cocci	10.2 (9.5)	16.6 (15.0)	3.5 (4.3)
Veillonella	10.2 (9.6)	16.6 (15.0)	3.5 (4.3)
+ve bacilli	0.4 (1.1)	21.2 (12.7)	62.8 (18.6)
Actinomyces	ND	21.2 (12.7)	58.9 (17.6)
Lactobacillus	0.03 (0.05)	0.05 (0.05)	0.05 (0.05)
-ve bacilli	1.3 (2.0)	4.0 (6.7)	2.7 (4.6)
Haemophilus	0.9 (1.5)	ND	ND
Bacteroides	ND	2.8 (4.0)	2.7 (4.6)

Table 6.11. cont.

Table 6.12. Total plaque flora expressed as percentage of total microflora, at 2, 7 and 21 days, from one subject, under each treatment condition, n=6.

NORMAL PLAQUE

	2 day	7 day	21 day
+ve cocci	92.4	32.4	25.6
S. mutans	0.03	0.03	0.04
S. sanguis	5.4	13.5	20.7
S. oralis	85.6	16.5	3.3
S. salivarius	ND	0.2	ND
S. milleri	0.7	ND	ND
Other Strep.	ND	0.8	ND
ANO_2 Strep.	0.2	0.8	2.4
Micrococcus/Staph	0.4	0.5	ND
-ve cocci	1.3	28.2	12.0
Veillonella	1.3	28.2	12.0
+ve bacilli	2.2	37.7	55.5
A. odontolyticus	2.2	23.3	13.4
A. v/n cat. +ve	ND	6.6	8.0
A. v/n cat. -ve	ND	6.3	33.9
A. meyeri	ND	ND	ND
Lactobacillus	ND	0.02	0.3
Bifidobacterium	ND	ND	ND
Eubacterium	ND	ND	ND
Unidentified	ND	1.5	ND
-ve bacilli	4.1	1.7	6.0
Haemophilus	3.9	0.8	0.8
Capnocytophaga	ND	ND	3.3
Bacteroides	0.2	0.8	1.7
Fusobacterium	ND	ND	ND
Unidentified	ND	ND	0.2

SUCROSE PLAQUE

	2 day	7 day	21 day
+ve cocci	76.3	55.4	46.0
S. mutans	0.1	0.1	0.1
S. sanguis	33.9	42.4	18.6
S. oralis	41.6	9.4	18.6
S. salivarius	ND	ND	ND
S. milleri	ND	0.6	ND
Other Strep.	ND	ND	3.0
ANO_2 Strep.	ND	0.6	5.9
Micrococcus/Staph.	0.8	2.3	ND
-ve cocci	17.1	12.2	6.3
Veillonella	17.1	12.2	6.3
+ve bacilli	4.1	26.7	42.3
A. odontolyticus	3.3	22.6	7.1
A. v/n cat. +ve	ND	2.1	4.8
A. v/n cat. -ve	ND	1.4	25.6
A. meyeri	0.4	ND	ND
Lactobacillus	0.02	0.02	1.6
Bifidobacterium	ND	ND	1.4
Eubacterium	ND	0.5	1.0
Unidentified	0.4	ND	0.8
-ve bacilli	2.5	5.7	5.4
Haemophilus	0.8	ND	ND
Capnocytophaga	0.8	1.1	1.8
Bacteroides	ND	2.7	3.0
Fusobacterium	ND	1.2	ND
Unidentified	0.9	0.7	0.6

Table 6.12. cont.

SPM

	2 day	7 day	21 day
+ve cocci	88.0	56.8	30.1
S. mutans	1.6	13.1	18.0
S. sanguis	21.4	27.4	6.2
S. oralis	62.7	16.0	5.6
S. salivarius	0.8	ND	ND
S. milleri	ND	ND	ND
Other Strep.	0.6	ND	ND
ANO_2 Strep.	ND	0.6	0.5
Micrococcus/Staph.	1.1	ND	ND
-ve cocci	10.2	16.6	3.5
Veillonella	10.2	16.6	3.5
+ve bacilli	0.4	21.2	62.8
A. odontolyticus	ND	14.5	14.3
A. v/n cat. +ve	ND	3.7	23.1
A. v/n cat. -ve	ND	3.0	28.4
A. meyeri	ND	ND	5.0
Lactobacillus	0.03	0.05	0.05
Bifidobacterium	ND	ND	4.7
Eubacterium	0.4	ND	ND
Unidentified	ND	ND	ND
-ve bacilli	1.3	4.0	2.7
Haemophilus	0.9	ND	ND
Capnocytophaga	ND	1.2	ND
Bacteroides	ND	2.8	2.7
Fusobacterium	ND	ND	ND
Unidentified	0.9	ND	ND

Table 6.12. cont.

Table 6.13. Mean (SD) microbial counts (log_{10} cfu per mm^2 enamel slab surface) of predominant isolates from plaque obtained from one subject, at 2, 7 and 21 days, under each treatment condition, n=6.

NORMAL PLAQUE

	2 day	7 day	21 day
+ve cocci	6.82 (0.28)	6.05 (0.63)	5.86 (0.61)
S. mutans	0.48 (0.68)	0.72 (1.05)	1.31 (0.73)
S. sanguis	4.99 (0.70)	5.60 (0.81)	5.59 (0.84)
S.oralis	6.80 (0.35)	5.74 (0.67)	5.05 (0.46)
-ve cocci	4.81 (0.77)	5.92 (0.27)	5.50 (0.17)
Veillonella	4.81 (0.77)	5.92 (0.27)	5.50 (0.17)
+ve bacilli	5.14 (0.78)	6.12 (0.46)	6.51 (0.48)
Actinomyces	5.14 (0.78)	6.11 (0.48)	6.26 (0.57)
Lactobacillus	0.04 (0.10)	0.23 (0.46)	1.53 (2.10)
-ve bacilli	5.35 (0.70)	4.74 (0.66)	5.50 (0.46)
Bacteroides	4.72 (0.66)	4.74 (0.66)	4.88 (0.54)
Total	6.84 (0.30)	6.50 (0.46)	6.50 (0.38)

SUCROSE PLAQUE

	2 day	7 day	21 day
+ve cocci	6.32 (0.94)	6.81 (0.52)	6.54 (1.13)
S. mutans	0.91 (0.84)	1.36 (0.90)	1.18 (0.96)
S. sanguis	5.92 (0.89)	6.70 (0.51)	6.15 (1.11)
S. oralis	5.96 (1.06)	5.94 (0.84)	5.98 (1.23)
-ve cocci	5.45 (0.46)	6.17 (0.46)	5.77 (1.11)
Veillonella	5.45 (0.46)	6.17 (0.46)	5.77 (1.11)
+ve bacilli	5.09 (1.02)	6.48 (0.48)	6.51 (1.18)
Actinomyces	5.09 (1.02)	6.46 (0.50)	6.45 (1.19)
Lactobacillus	0.26 (0.44)	0.09 (0.07)	2.60 (2.82)
-ve bacilli	4.80 (1.05)	5.77 (0.43)	5.69 (1.22)
Bacteroides	4.63 (0.86)	5.60 (0.48)	5.45 (1.12)
Total	6.42 (0.87)	7.04 (0.44)	6.85 (0.89)

SPM

	2 day	7 day	21 day
+ve cocci	5.95 (1.05)	6.52 (0.68)	6.36 (0.58)
S. mutans	3.40 (1.40)	5.60 (0.89)	5.57 (1.24)
S. sanguis	5.21 (1.07)	6.17 (0.54)	5.57 (0.82)
S. oralis	5.73 (1.14)	5.79 (1.03)	5.48 (0.74)
-ve cocci	4.76 (0.58)	5.95 (0.14)	5.47 (0.65)
Veillonella	4.76 (0.58)	5.95 (0.14)	5.47 (0.65)
+ve bacilli	4.10 (0.86)	6.09 (0.34)	6.70 (0.36)
Actinomyces	4.01 (0.86)	6.09 (0.34)	6.67 (0.37)
Lactobacillus	0.47 (0.56)	1.15 (1.32)	2.03 (1.07)
-ve bacilli	4.28 (1.10)	5.33 (0.77)	5.35 (0.61)
Bacteroides	4.01 (0.86)	5.30 (0.72)	5.35 (0.61)
Total	5.95 (1.01)	6.78 (0.39)	6.87 (0.40)

Table 6.13. cont.

PERCENTAGE COUNT

	Time	Treatment	Position	Side
+ve cocci	***	NS	NS	NS
S. mutans	NS	***	NS	NS
S. sanguis	NS	**	*	NS
S. oralis	***	NS	NS	NS
-ve cocci	*	NS	NS	NS
Veillonella	*	NS	NS	NS
+ve bacilli	***	NS	NS	*
Actinomyces	***	NS	NS	*
Lactobacillus	NS	NS	NS	NS
-ve bacilli	NS	NS	NS	NS
Haemophilus	*	*	NS	NS
Bacteroides	*	NS	NS	NS

NS = Not significant ; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

Table 6.14. Statistical analysis of effect of time, treatment, position and side on percentage predominant cultivable flora, from plaque obtained from one subject, at 2, 7 and 21 days.

ABSOLUTE COUNT

	Time	Treatment	Position	Side
+ve cocci	NS	NS	NS	NS
S. mutans	*	***	NS	NS
S. sanguis	*	*	NS	NS
S. oralis	NS	NS	NS	NS
-ve cocci	***	NS	NS	NS
Veillonella	***	NS	NS	NS
+ve bacilli	***	NS	NS	NS
Actinomyces	***	NS	NS	NS
Lactobacillus	**	NS	NS	NS
-ve bacilli	NS	NS	NS	NS
Bacteroides	**	NS	NS	NS
Total	NS	NS	NS	NS

NS = Not significant ; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$

Table 6.15. Statistical analysis of effect of time, treatment, position and side on microbial counts of predominant cultivable flora, from plaque obtained from one subject, at 2, 7 and 21 days.

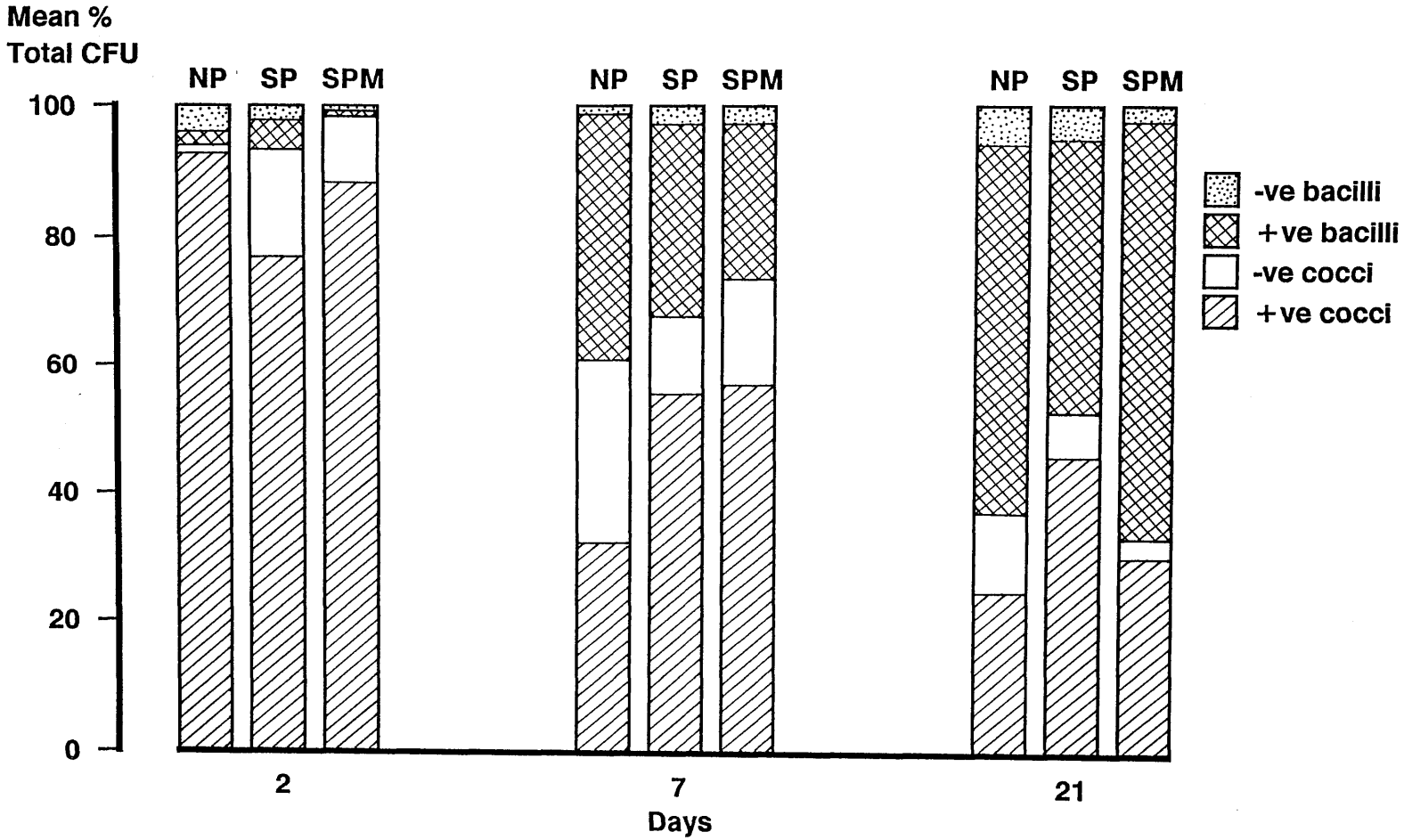


Fig. 6.7. Mean percentage distribution of Gram positive and negative cocci and bacilli, under each treatment condition, at 2, 7 and 21 days, n=6.

6.4. Discussion.

6.4.1. Two-day Study.

Comparison of Normal and Sucrose Plaque Microflora.

The sampling times used in this study were chosen to allow the effect on the plaque microflora of implantation of the Strep. mutans on to the test areas to be assessed, and also to allow a comparison of the results with the findings of other studies on early plaque development. In the present investigation, it was possible to monitor the change with time in absolute counts of the organisms present in the plaque, as well as expressing each as a percentage of the total microflora. While some other studies have also related the counts to unit surface area of enamel (Socransky et al., 1977; Nyvad & Kilian, 1987), others have expressed the results only in proportional terms (Ritz, 1967; Theilade et al., 1982).

Although the studies by Socransky and co-workers (1977) and Nyvad and Kilian (1987) reported the total count of microorganisms as well as the proportional counts of the isolates, absolute counts for each organism were not given. In the present study, the number of each organism isolated was expressed as a proportional count, and, for the predominant organisms, the absolute count was also calculated. In early plaque, the total count is changing over the experimental period, and therefore if a change in relative proportions of organisms is observed, it is important to know whether this is due to an increase in the numbers of some organisms, a decrease in others, or a combination of both. Only if proportional and absolute counts of organisms are known, can the microflora of early plaque be fully assessed.

The use of multiple enamel slabs located within a small area on the in situ appliance, meant that longitudinal studies could be performed, with the same intra-oral conditions pertaining throughout the experiment. Thus, when plaque samples were removed from the different enamel slabs, after varying time intervals, subject characteristics such as salivary microflora would be as similar as possible at the different sampling times. This is in contrast to the work of Ritz (1967) and Socransky and co-workers (1977), where plaque sampling after varying intervals from base-line was

performed on sequential occasions, and thus changes in the oral environment between the experimental runs may have influenced the microbial results obtained.

The results of the total counts for the three subjects combined showed an increase in microbial numbers from 2 to 48 hours, under both normal and sucrose plaque conditions. This was most evident between 6 and 30 hours in both groups, with a smaller increase to 48 hours under NP conditions, and the count remaining stable between 30 and 48 hours under SP conditions.

Nyvad and Kilian (1987), who used an appliance model with human enamel blocks mounted in the lower vestibular region, found an exponential increase in cell numbers between 0 and 8 hours, and a slower increase thereafter to 24 hours, at which time a count of 10^6 - 10^7 cfu/0.024 cm^2 was found. Socransky and co-workers (1977) used the buccal surface of the upper first molars and found a baseline value following cleaning of 10^3 cfu/cm^2. After 5 minutes this count increased to 10^5 - 10^6 cfu/cm^2, and thereafter remained stable up to 8 hours. Between 8 hours and 1 - 2 days a sharp increase was found, up to a total of 10^8 cfu/cm^2 tooth surface. The results of the present study are perhaps more similar to those of Socransky and co-workers, since the total counts remained fairly stable between 2 and 6 hours (10^2 - 10^3 cfu/mm^2 - ie, 10^4 - 10^5 cfu/cm^2) and rose to 10^4 - 10^6 cfu/mm^2 (ie, 10^6 - 10^8 cfu/cm^2) by 1 - 2 days.

Extra-oral sucrose application had a significant effect on viable counts, with an increase in number associated with SP conditions being apparent up to 30 hours, but little difference being seen at 48 hours. Nyvad & Kilian used sucrose supplementation only during the initial 8 hours, during which time the exponential increase in count was seen. However, in the present study, although increased numbers of organisms were seen in the sucrose plaque, no obvious difference in the pattern of increase with time was apparent between the normal and sucrose plaque conditions.

The proportional results showed that, under both conditions, positive cocci predominated in all samples between 2 and 48 hours, ranging throughout from a mean of 66.5 to 88.2 %. No significant difference was found

between the proportions under NP and SP, but overall a significant time effect was seen, ($p < 0.05$), with the highest levels of positive cocci being found between 6 and 30 hours.

Streptococci comprised almost the total positive cocci in all samples, although members of the Micrococcus / Staphylococcus group comprised 8.3 % of the total flora at 2 hours under NP conditions. This may have been a result of skin contamination, although some other studies have found a similar proportion of this group of organisms in a few early plaque samples (Socransky et al., 1977; Nyvad & Kilian, 1987). Of the streptococcal species, Strep. oralis dominated the plaque microflora at almost all sampling times, with the mean percentage of the total cfu at each sampling time ranging from 35.0 - 78.9 %. The highest levels of this species were again found between 6 and 30 hours, and while Nyvad and Kilian also found an increase in proportion from 4 to 24 hours, the percentage levels were much lower than in the current study. However, the problems with nomenclature (previously discussed in Chapter III) make comparison of levels of the streptococcal species reported in different studies extremely difficult - for example, some authors have reported that Strep. sanguis predominates in early plaque samples (Ostrom et al., 1977; Socransky et al., 1977; Syed & Loesche, 1978), while Nyvad and Kilian (1987) found Strep. mitis to be present in greatest proportion. They suggest, however, that the variation in the reported results may have been due to differences in the nomenclature used, particularly with regard to the Strep. sanguis / mitis / oralis group.

In the present study, the highest proportions of Strep. salivarius were found in the 2 hour samples (5.2 % (NP) and 10.5 % (SP)), while the levels found under both treatment conditions at 48 hours were under 1 %. Socransky and co-workers (1977) found that Strep. salivarius was not detected in any plaque sample after 15 minutes, while Theilade and co-workers (1982) isolated this organism in only very small proportions from a few 3 and 8 hour samples from the gingival crevice area of the natural dentition. Strep. salivarius comprised a much larger percentage of the total flora in the study by Nyvad and Kilian (1987), with the median value at 4 hours being 16 %, falling to a level of 4 % at 24 hours. In the latter study the enamel blocks were mounted on a buccal flange in the lower vestibulum, and the relatively high numbers of Strep. salivarius seen

in the initial samples may have been due to direct contact between the enamel surface and the buccal mucosa, allowing direct inoculation of the organism in the test plaque. In the present study, however, the enamel slabs were mounted on recessed troughs in the lingual flanges of a lower appliance with no direct mucosal contact. The comparatively high levels of this organism in the initial plaque samples of both studies may reflect the fact that Strep. salivarius comprises a relatively high proportion of the salivary microflora, and therefore will account for a relatively high percentage of the organisms associated with the enamel surface, when it is newly exposed to the oral environment. As salivary organisms may be loosely bound contaminants, rather than firmly adherent members of the plaque flora, flushing of the enamel surfaces with sterile saline was performed in this study (see Section 2.5.1.) prior to sampling, as recommended by Nyvad and Kilian (1987).

Strep. salivarius is not normally found in dental plaque (Rogers, 1969), and the proportions of this organism found at 48 hours in this study were lower than the initial levels, suggesting that other members of the oral microflora were becoming adsorbed and replicating during this period, resulting in a gradual exclusion of the Strep. salivarius cells from the plaque. Ostrom and co-workers (1977) found Strep. salivarius comprised approximately 50 % of the total streptococcal flora after 7 days, in their intra-oral cariogenicity test model. The authors stated that this was probably due to the Dacron gauze covering the enamel slabs providing a suitable retentive surface for this organism, with the extra-oral sucrose applications which were employed also contributing to the rise in numbers of Strep. salivarius. In the present study, although extra-oral sucrose exposures were carried out, the slabs were uncovered during the experimental period, and while the Strep. salivarius counts up to 10 hours were higher in the sucrose associated plaque, the levels in both plaques fell after this time, to similar low levels.

Actinomyces spp. accounted for the majority of positive bacilli isolated from all samples. At two hours, relatively high levels of Gram positive bacilli were present, namely 10.7 % in normal plaque where the catalase negative division of A. viscosus / naeslundii predominated, and 13.5 % in sucrose plaque, in which A. odontolyticus and catalase positive and negative divisions of A. viscosus / naeslundii were present in similar

proportions. Nyvad and Kilian (1987) found similar levels of Gram positive bacilli at the initial sampling time (4 hours) but of these organisms only 50 %, approximately, were ascribed to the genus Actinomyces, with the A. viscosus / naeslundii group predominating and the majority of the remaining positive bacilli being unidentified due to insufficient growth or indistinct or incomplete results. In a study by Theilade and co-workers (1982), Gram positive bacilli formed 11 % of the total isolates in the 3 hour samples, with the vast majority being identified as A. viscocus-like. Similarly, Socransky and co-workers (1977) found A. viscosus to predominate in early plaque samples isolated from a smooth tooth surface, in one individual. In the present study, however, A. viscosus-like organisms did not form a greater proportion of the plaque microflora than A. naeslundii-like organisms, in the initial samples, and Nyvad and Kilian comment that although in vitro studies demonstrate differences in the ability of A. viscosus and A. naeslundii to adhere to pellicle-covered hydroxyapatite surfaces, the characteristics of different individuals may cause varying results in vivo.

In the current study, there was a decrease in proportion of Actinomyces from the initial samples until 10 hours, when the lowest value was found in SP plaques (2.8 %) and the organism was undetectable in normal plaques. Thereafter, the proportion in both NP and SP increased in the 24 hour samples, fell again by approximately 50 % at 30 hours, and increased to around the initial level at 48 hours. Nyvad and Kilian (1987) found similar results, with the lowest mean percentage count (approximately 4 %) being seen at 8 hours, and an increase at 24 hours, although the count at this time was still lower than the initial count. Scoransky and co-workers (1977) reported a decrease in the proportion of Actinomyces spp. at 1 - 2 days, which they attributed to a rise in Strep. sanguis, as the absolute counts of Actinomyces spp. remained unchanged. Nyvad and Kilian also noted a rise in proportion of streptococci accompanying the fall in percentage count of positive bacilli, with the predominant streptococcal species being Strep. oralis - a similar pattern was seen in the current study.

Socransky and co-workers (1977) suggested that the change in relative proportions of streptococci and Actinomyces spp. may be due to earlier adsorption to the enamel surface or earlier replication of the streptococci

while Nyvad and Kilian (1987) considered that the relative increase in streptococci was due to differences in growth rates of the two genera. In the present study, the absolute count of Actinomyces spp. fell between 2 and 6 hours, but by 10 hours was present in considerably greater numbers than in the initial samples. However, the proportion of Actinomyces spp. was at a minimum at 10 hours. Positive cocci were more abundant in the initial sample than Actinomyces spp., and rose in absolute terms between 2 and 6 hours, and between 6 and 10 hours. From 10 hours onwards, the numbers of positive cocci and Actinomyces spp. increased at similar rates, although the former were always present in greater number. These findings would be in keeping with the theory put forward by Socransky and co-workers, suggesting that Actinomyces spp. take longer to adsorb and commence replicating on the enamel surface.

Gram negative cocci increased in mean proportion up to 10 hours in normal plaque, and 24 hours in sucrose plaque, before falling but rising again in the final sample. Neisseria spp. had its highest proportion in the 24 hour plaques, where it accounted for 4.5 % (NP) and 10.2 % (SP). Horikawa and co-workers (1978), using a selective medium for Neisseria spp., found that the highest levels (approximately 10 %), of this organism occurred at one hour, with the mean proportion decreasing at 4 and 12 hours. The mean percentage of Veillonella spp. found in the current study, ranged from 0.5 % to 12.7 %, with the highest values being seen at 10 hours (NP), 24 hours (SP) and 48 hours (NP and SP). Ritz (1967) reported that Veillonella spp. accounted for 1 % of the total microflora at 1 day, compared with 0.5 % (NP) and 3.5 % (SP) in the present study. Nyvad and Kilian found that negative cocci comprised less than 2 % of the isolates at all sampling times up to 24 hours, while Theilade and co-workers (1982) quoted a rise in median proportion from 0 % at 3 hours to 22 % at one day. It is suggested that the frequently reported increase in Veillonella spp. and decrease in Neisseria spp., with time, may be due to changes in the local plaque environment, which becomes more anaerobic with increasing plaque thickness (Ritz, 1967; Horikawa et al., 1978).

Negative bacilli remained fairly stable in normal plaque, accounting for 6.6 to 10 % of the total microflora at all sampling times, while the proportion of this group of organisms, under SP conditions, accounted for approximately 7 % of organisms at 2 - 22 hours, but decreased to 2.6 % at

48 hours. Gram negative rods were isolated only rarely from plaque samples by Nyvad and Kilian (1987), while Theilade and co-workers (1982) found that this group comprised 3 % of the organisms in Gram stained smears at 24 hours. Kilian and co-workers (1976) suggested that Haemophilus spp. may be a common organism in early plaque, and found that this genus comprised approximately 10 % of the total anaerobic microflora in 18 hour plaque samples from smooth tooth surfaces, while, in the present study, Haemophilus spp. ranged from ND to 6.7 %, with no trend being seen in relation to time.

According to the results of other studies, Bacteroides spp. do not appear to be common organisms in early plaque samples (Socransky et al., 1977; Theilade et al., 1982; Nyvad & Kilian, 1987). However, in the current study, while Bacteroides spp. were undetectable or isolated in very low proportion in plaque samples from two of the subjects, in Subject A, this organism accounted for all the negative bacilli in the normal plaque samples (ranging from 1.9 to 12.2 %). When the results for the three subjects were combined, the mean percentage of Bacteroides spp. was still relatively high, reflecting the wide inter-subject variation occurring in this small subject sample size. However, in the 48 hour sample from the 21 day study, the finding of relatively high levels of Bacteroides spp. in Subject A was not repeated, suggesting that the results from the early study were uncharacteristic.

The importance of obtaining data in both proportional and absolute counts can be seen by considering the results for individual groups and organisms. For example, while the proportion of most organisms was unaffected by time, or only a slight association was seen, a highly significant rise in the numbers of total organisms and each group, genus and species was observed. Similarly, while the application of extra-oral sucrose had little effect on the proportions of most of the isolates, a comparison of absolute counts obtained from normal and sucrose plaques demonstrated that sucrose stressing caused a significant increase in the number of most organisms. Conversely, while the proportion of Strep. sanguis was significantly higher under normal plaque conditions, the actual counts of the organisms in plaque obtained under the two treatment conditions was similar.

Thus, important trends in relation to the plaque microflora may not be appreciated if results are given with respect to only the proportional or absolute counts.

Effect of Strep. mutans Implantation on Early Plaque Samples.

As inoculation of Strep. mutans was performed only in Subject A in this study, the results of the SPM plaques can only be compared with the NP and SP samples obtained from that subject, rather than the combined results of all three subjects.

While Strep. mutans was not detected in any of the plaque samples obtained under NP and SP conditions, the organism comprised 11.0 % of the total isolates from the SPM plaque at 2 hours. In the 6 hour SPM samples, Strep. mutans accounted for 28.1 % of the microflora, but fell to an undetectable level at 10 hours, and thereafter was present at 0.1 % of the microflora at each sampling time.

The initial high levels of Strep. mutans were probably due to implantation of the organism at 0 and 4 hours, although further inoculation was performed at 24 and 28 hours, with no subsequent rise in proportion of the organism isolated occurring in the 30 and 48 hour samples. This suggests that successful implantation only took place where a microflora was not already established on the enamel, and that other organisms, derived from saliva, competed with the Strep. mutans and became dominant within 10 hours, although the latter continued to be isolated in all 24 hour samples. Further implantation of Strep. mutans did not cause a rise in proportion of this organism, presumably due to the fact that the other organisms were already established, and may, in addition, have been producing factors antagonistic towards this organism.

The absolute counts of Strep. mutans at 30 and 48 hours were considerably higher than in all other samples including those in which the organism comprised the highest proportional count, suggesting that multiplication of this organism was taking place throughout the experimental period, but to a lesser extent than with other plaque organisms.

In the two hour samples under SPM conditions, the proportion of positive bacilli, at 1.8 %, was lower than in NP and SP samples (23.4 and 20.0 %, respectively), while similar low counts (less than 5 %) were seen at 6 hours, under all treatment conditions. At this sampling time, the major difference in mean percentage count, other than with Strep. mutans, was with Strep. oralis which comprised 43.5 % in SPM compared to 75.0 % and 77.0 % in normal and sucrose plaques, presumably due to the presence of large numbers of Strep. mutans in the total microbial count. However, despite the relatively low proportion of Strep. oralis in SPM samples at 6 hours, the actual count of this organism was greater than the count in NP samples, where the species accounted for 75.0 % of the total flora.

6.4.2. Twenty-one Day Study.

The results of the 21 day study showed a significant decrease in the relative proportions of streptococci and an increase in the levels of positive bacilli over the experimental period, under each treatment condition. This is in agreement with the in vivo findings of Syed and Loesche (1978), where a change in the predominant genus from Streptococcus to Actinomyces was observed over a three-week period in inter-proximal plaques. In the current study, the fall in the streptococcal count with time was due largely to a marked decrease in the proportion of Strep. oralis, and a rise in the positive bacilli, due mainly to a significant increase in Actinomyces spp. At 7 days, A. odontolyticus was the predominant species in this group, while in the three-week samples, the catalase negative division of A. viscosus / naeslundii (ie. A. naeslundii-like) comprised the major proportion of Actinomyces spp. in all three treatment groups. Similarly, Socransky and co-workers (1977) found an increase in A. naeslundii-like organisms with time, during a 16-day experimental period.

The proportion of Strep. mutans was unchanged at 0.1 % or less in normal and sucrose plaques at all sampling times, while under SPM conditions, the mean percentage count of this organism was 1.6 % in the 2 day samples rising to 18.0 % at 21 days. As no implantation of Strep. mutans was performed after 28 hours, this suggests that the proportion of the organism present at two days was sufficient for it to be selected for under the sucrose conditions. However, no such increase in proportion

was seen in samples where extra-oral sucrose applications were performed without implantation of Strep. mutans, suggesting that, in this subject, the concentration of Strep. mutans was below the threshold necessary for the organism to become a dominant member of the plaque flora, even when regular sucrose applications were used. In terms of absolute counts of organisms, Strep. mutans increased by a similar amount over the 21 days in normal and sucrose plaques, but only under SPM conditions was a marked rise seen.

Strep. salivarius was very rarely detected in all treatment conditions, and where isolated, formed a very low percentage of the total microflora. This is in agreement with the findings of other studies described in Section 4.6.9., where this organism has been found to be a rare constituent of mature plaque.

Implantation of Strep. mutans has been performed by a number of workers, but only early samples have been collected, to determine the success of the colonisation by this organism (Svanberg & Loesche, 1978; Svanberg & Krasse, 1981). No studies reporting longterm changes in the plaque microflora following inoculation with Strep. mutans are available for comparison with the current results. An appliance study by Borden and co-workers (1980) involved seeding of Strep. mutans cells on Dacron covered enamel slabs and application of sucrose extra-orally. Although as previously described (see Section 4.6.8.) the methodology employed differed from that used in the current study, and the two experiments are therefore not directly comparable, Borden and co-workers did investigate the microflora of plaque samples over a 7-day period, following implantation. They found that Strep. mutans comprised a higher proportion (58 %) of the streptococcal plaque flora than was found in the present study (23 %). Higher levels of Strep. salivarius (17 %) were also found in the experimental plaque of Borden and co-workers, and the very high proportions of both these streptococcal species was thought to be due to the Dacron gauze providing a suitable retentive meshwork in which these organisms could proliferate. In the present study, as no Dacron covering was used on the enamel slabs, organisms present initially, such as implanted Strep. mutans, were required to adhere and compete with other plaque organisms, without the highly retentive framework provided by the gauze. This would explain, at least in part, the lower Strep. mutans

levels found in the current study, compared to those reported by Borden and co-workers.

Negative cocci formed a significantly higher proportion of the plaque microflora in the 7-day samples, compared to the 2- and 21-day samples where similar proportions were found. Only Veillonella spp. were isolated, and this absence of other negative cocci was also reported by Socransky and co-workers (1977). Their results were comparable with the current study, the highest proportion of Veillonella spp. occurring at 8 days, with a fall to undetectable levels at 16 days. Although a progressive increase in Veillonella spp. was not seen with time, the proportions of some other anaerobic species, such as anaerobic streptococci showed a slight rise in the more mature plaque samples.

Lactobacillus spp. were either undetected or were isolated in low proportion from all samples under all treatment conditions, despite the relatively high levels of Strep. mutans found in the SPM plaques, which would be expected to produce high levels of acid, and therefore possibly select for this aciduric organism.

Haemophilus spp. comprised a low proportion of the microflora in the 2-day plaque samples, and thereafter fell to lower, or undetectable levels, in all treatment groups. A fall in the proportion of Haemophilus parainfluenzae, the only species of Haemophilus isolated in this study, has previously been reported in maturing plaque samples (Kilian et al., 1976).

The proportion of Bacteroides spp. was 3 % or less in all plaque samples, but increased significantly with time in plaque samples obtained under all treatment conditions, as might be expected for a strict anaerobe such as this organism, as discussed below (Section 6.4.3.).

While a marked change in the microbial composition of plaque samples was observed at 2, 7 and 21 days, there was no significant increase, overall, in the total microbial count with time, although a moderate rise occurred in the SPM samples. However, the difference in microbial counts, when comparing the three treatment conditions was not significant. The similarity of total bacterial counts in these plaques of different age, is comparable to the work of Socransky and co-workers (1977) and Syed and

Loesche (1978), who found no change in absolute counts in plaque samples aged 2 - 16 days and 7 - 21 days, respectively, with Socransky and co-workers stating that a maximal bacterial mass is reached in plaque, within which the microbial composition is continually changing.

6.4.3. Microbial Composition of Plaque from Two Hour to Twenty One Days, in One Subject.

The results of this study demonstrated a rise in the total microbial counts in plaque samples up to 30 - 48 hours, after which the total number of organisms remained relatively static. The maximum number of organisms per unit surface area of enamel was similar in all treatment groups, but this value was achieved sooner in sucrose plaque, possibly due to the sucrose allowing more rapid replication to take place. However, the plateau level of counts was reached at 48 hours with both SPM and NP samples, suggesting that the implantation of Strep. mutans interfered with the replication of normal early colonisers, so that the enhanced multiplication seen with sucrose did not occur.

After a level of approximately 6.7 $\log_{10}$ cfu per mm^2 enamel surface was reached, under each treatment condition, no further increase in total numbers of organisms was seen, suggesting that the capacity of the site had been reached. Sheer forces (Marsh & Martin, 1984) and nutrient availability may limit the bacterial mass which the plaque can attain.

A change in the microbial composition of plaque was observed over the three weeks, with streptococci predominating initially but being replaced by Actinomyces spp. as the predominant organism between 7 and 21 days. The replacement of the pioneer species with time, to a climax community with a more complex microflora and high species diversity, takes place due to changes in the local environment during plaque accumulation, with alteration in the oxidation-reduction potential, pH, availability of nutrients and production of metabolic end-products, selecting for different organisms (Hardie & Bowden, 1976). Furthermore, "secondary" plaque formers may adhere to organisms already present in the plaque, and therefore accumulate only at a later stage (Socransky et al., 1977). Such population changes due to microbial factors are an example of autogenic succession (Marsh & Martin, 1984).

In the samples where inoculation of Strep. mutans had been carried out, the organism was isolated in relatively high proportion at 2 and 6 hours, but was undetectable or present in very low proportion thereafter until 48 hours. However, in the 21 day experiments, performed at different times from the 2 day runs, only a low proportion (1.6 %) of Strep. mutans was found in the initial samples, but a considerable increase in mean percentage count occurred at the 7 and 21 day sampling times, associated with a rise in mean count from 3.40 $\log_{10}$ cfu per mm^2 enamel surface to 5.57, over the 21 day period. In the 2-day experiment, the proportional count was only 0.1 % at 48 hours, and the absolute count was 2.73, representing only a fifth of the equivalent count at 48 hours in the longer experiment. As has already been discussed, it seems that a threshold count may exist for Strep. mutans, and only if this is exceeded, and sucrose is present, does a significant rise in the count occur. Therefore, it is uncertain whether the count obtained at the end of the 2-day study would have been sufficient for the organism to become a significant member of the plaque microflora. Thus, although by compiling the data from the two experimental runs involving the one subject, some idea of the progression of counts over the 3 week period could be obtained, the comparison of the results at the different times is not as valid as would have been the case if all the samples had been obtained during the one experimental run.

6.5. Conclusions.

The methodology employed in the studies detailed in this Chapter allowed plaque sampling from enamel slabs of known surface area to be performed, thus permitting isolates to be expressed both as proportional and absolute counts, so that the effect of time on microbial composition and bacterial counts could be investigated. Very few studies have previously reported the microbial composition of early plaque with regard to both proportional and absolute counts. The current study demonstrated the importance of both, so that an accurate indication of the change in the numbers and dominance of different organisms can be assessed, in relation to time and treatment conditions.

The use of multiple enamel sites on an in situ appliance allows longitudinal studies to be performed, with plaque samples obtained after

varying time intervals from baseline, so that the local environment is as constant as possible throughout the experimental period.

The application of sucrose during the development of plaque made little difference to the proportional microbial composition, but resulted in higher counts of all organisms in very early plaque.

The use of the in situ device allowed slabs on the two sides of the appliance to be subjected to different treatment conditions simultaneously, thus ensuring that the same intra-oral conditions were affecting all the slabs. The fact that counts of Strep. mutans were not increased, under normal and sucrose plaque conditions, when the organism was implanted on the contra-lateral side, indicated that no cross-over effect occurred, and suggested that valid comparisons between the two sides, under very similar intra-oral conditions but different treatment conditions, could be made.

These preliminary studies, involving three subjects in the 2-day investigation and one subject in the 21-day study, showed similar findings with regard to microbial composition and total bacterial counts, compared to previous work. However, the appliance has advantages over other experimental models, as described above, in relation to the possibility of obtaining absolute as well as proportional counts, longitudinal studies using multiple sites within a small area and the ability to subject the two sides to different treatment conditions simultaneously, allowing comparative studies to be made. This suggests that the appliance may be a suitable model for more extensive investigation of the microbial composition of smooth surface plaque, and further, that by applying test substances to one side of the appliance, with the other, untreated side, acting as the control, this model may be used for the investigation of the effect of anti-microbial agents on plaque microflora.

CHAPTER VII

CONCLUDING DISCUSSION.

7.1. Introduction.

As discussed in Chapter I, dental caries is a disease in which many inter-relating factors are involved, with the three principal variables being the host, the diet and the plaque microflora. This thesis was mainly concerned with the relationship between the composition of the plaque microflora and the mineral status of the associated enamel surface. In addition, the studies undertaken allowed many other factors, known to influence the carious process, to be investigated to varying extents. This Chapter therefore inter-relates the results of the different experiments carried out in this thesis, and suggests where further work in this field could be pursued.

7.2. Variation in Enamel Susceptibility.

Dental enamel is not homogeneous, and differences in the susceptibility of enamel sites to standard acid attack have been reported (de Groot *et al*, 1986). Almost all investigations on the relationship between plaque microflora and enamel demineralisation, carried out in the human oral environment, have involved cross-sectional or longitudinal clinical studies in which the subjects' natural dentition is examined. In such investigations, it is impossible to determine the susceptibility of the enamel tooth sites to demineralising stresses, and many studies fail to discuss this important variable when relating plaque microflora to demineralisation.

The use of the appliance model involving thin enamel sections, allowed an *in vitro* investigation to be carried out on the susceptibility of control sections from teeth which were to be used in the main *in situ* demineralisation experiment. The results described in Section 3.5.3. agreed with other *in vitro* reports, in finding large variation in susceptibility between different sites. There was no significant difference between mean levels of demineralisation in sections from different teeth, suggesting that at least as much variation exists in enamel susceptibility

between different sites on the same tooth, as between teeth. Greater amounts of demineralisation were seen at the cervical margin compared to other sites, so the former area was not used as a test site in appliance studies described in Chapters III and IV.

It has been suggested that removing the outer surface layer of enamel pre-experimentally may increase enamel susceptibility and reduce variation in observed demineralisation. Abraded and non-abraded enamel sections from the same tooth were therefore included in the in vitro experiment detailed in Chapter III, and were also used in an in situ investigation described in Chapter IV. Both studies found similar results, namely that abrasion of the enamel surface resulted, overall, in more experimental mineral loss, but that the variation between abraded enamel sites was just as great, if not greater, than in non-abraded surfaces. The variation in demineralisation found in the in situ study could not be explained by the composition of the overlying plaque microflora.

One of the aims of carrying out this small in situ study was to determine whether treating the enamel surface in this manner affected the composition of the associated plaque microflora, as this does not appear to have been investigated previously. No difference was found between abraded and non-abraded enamel, with regard to either proportional or absolute counts of the associated plaque microorganisms, and in this respect, abraded enamel would appear to be suitable for investigations of the microbiology of early enamel demineralisation. However, the variation in susceptibility of enamel was not overcome by removing the enamel surface, and so it is still uncertain at present whether or not abraded enamel should be used in such studies. Thus, variation in susceptibility of different enamel sites is one of the factors involved in the initiation of caries which it is impossible to control completely, and this must be borne in mind when interpreting the results of studies involving the relationship between plaque microflora and enamel demineralisation. Further, this may have been one of the factors responsible for the poor correlation between numbers of acidogenic organisms and quantity of mineral loss, found at some sites in the in situ demineralisation study described in Chapter IV.

7.3. Quantification of Enamel Demineralisation.

Most studies attempting to relate plaque microflora to enamel demineralisation have been performed *in vivo*, where identification of incipient carious lesions on the natural dentition is difficult, and accurate quantification of the mineral loss is not possible. A few studies have therefore employed enamel slabs mounted on an intra-oral appliance, the use of which allows accurate assessment of mineral content to be performed post-experimentally (Ostrom *et al.*, 1977 ; Gallagher & Pearce, 1979). However, pre-experimental quantification of mineral content cannot be undertaken, and therefore adjacent unexposed enamel sites are used as a control, in assessment of change in mineral content during the experimental period. Bovine enamel slabs were employed in the *in vitro* studies described in Chapter V, and in these experiments assessment of demineralisation was carried out as described above. The suspicion that this does not give a true indication of mineral loss was strengthened by the fact that the results obtained with this method were least in keeping with the overall results of the four methods used to assess the cariogenic potential of *Strep. mutans*.

The use of the single-section technique in the *in situ* demineralisation study allowed both pre- and post-experimental quantification of mineral content, so that mineral loss during the experimental period could be calculated. Thus, the most accurate assessment of enamel demineralisation is achieved by using thin enamel sections. Although using proportional counts, the microbial composition of the plaque associated with the enamel sections was less similar to natural tooth plaque than was slab plaque, the microflora of the plaque was qualitatively similar in all cases. However, quantification of the mineral content of slabs mounted on the appliance was not possible either pre- or post-experimentally, as described in Chapter IV. While modification of the appliance design would allow the use of thicker slabs and permit post-experimental sectioning and microradiography of the enamel to be carried out, this would still not allow experimental mineral loss to be measured accurately, as the only control available would remain sites of adjacent unexposed enamel. Thus, at present, only by using enamel sections, which are associated with a microflora qualitatively similar to that obtained from the

natural dentition, can mineral loss occurring during the experimental period be quantified accurately.

7.4. Diet and Enamel Demineralisation.

Since in vivo studies have found an association between plaque sucrose exposure and levels of demineralisation, stressing of test sites with a 10 % sucrose solution was carried out in two of the experimental protocols as described in Chapter IV. The use of an appliance model enabled the sugar to be applied extra-orally, thus overcoming the ethical problem of attempting to create cariogenic conditions in the human oral environment.

The results showed no significant difference in mean proportions or absolute counts of microorganisms between normal and sucrose plaques, but found a higher isolation frequency of Strep. mutans associated with the latter protocol. The differences in plaque composition were not as marked as those reported by other workers, probably due to the fact that, in the current study, no attempt was made to control the normal background diets of the individuals during the experimental periods. However, the early plaque colonisation study (Section 6.3.1.) found higher total absolute counts of plaque microorganisms in sucrose plaque, compared to normal plaque, during the first 30 hours. By 48 hours, similar microbial counts were obtained under both conditions, suggesting that the addition of sucrose allowed a faster rate of plaque growth, until the maximum cell mass had been achieved.

The enamel sites associated with sucrose stressing in the in situ experiments outlined in Chapter IV, experienced slightly higher levels of demineralisation than those under normal plaque conditions, and this finding is in agreement with those of other studies (von der Fehr et al., 1970).

As discussed in Section 4.6.2., diet analysis is extremely difficult, and therefore detailed analysis of the diet histories of the individuals was not attempted. However, no correlation was seen between frequency of intake of fermentable carbohydrate during the experimental periods and levels of in situ demineralisation.

7.5. Relationship Between Plaque Microflora and Demineralisation.

7.5.1. Advantages of Appliance Model.

The main aim of the studies in this thesis was to develop an in situ model suitable for investigating the relationship between the microbial composition of plaque and mineral loss in the associated enamel.

Previous in vivo investigations have mostly involved cross-sectional and longitudinal studies. While these have generally shown an association between the initiation and development of caries and Strep. mutans levels, and in some cases with lactobacilli, cross-sectional studies are unable to demonstrate a cause-and-effect relationship (Edwardsson, 1986). Whilst longitudinal studies allow monitoring of the progression of a site from intact surface to carious lesion, such investigations have practical disadvantages, notably the requirement for a very large number of sites to be studied over a long time interval, with consequent demands on human, laboratory and financial resources. The caries-prone sites in the natural dentition are fissures and interproximal areas, from which accurate plaque sampling is virtually impossible. Therefore, correlation between a discrete lesion and its associated plaque microflora is not possible. Furthermore, problems arise in attempting to detect early lesions on plaque-covered teeth, and even after detection, accurate quantification of the incipient lesion cannot be performed.

The use of an intra-oral appliance on which enamel specimens are mounted can overcome some of these difficulties, as accurate plaque sampling and quantification of enamel loss at discrete sites are possible, and rapid mineral loss can be achieved by stressing test sites without affecting the subject's dentition. However, only a few studies have reported the use of an appliance model in this context (Ostrom et al., 1977 ; Gallagher & Pearce, 1979), and, as these have employed Dacron-covered enamel slabs, microbial composition of the plaque was different from that on uncovered enamel surfaces, and accurate plaque sampling from specific areas was not possible. The appliance model used in the present study was designed to allow the use of uncovered enamel sections and slabs, from which accurate plaque sampling could be achieved.

Absolute microbial counts, per mm^2 enamel surface, could be determined by sampling the plaque from enamel slabs, and this was utilised in the study on early plaque development, described in Chapter VI, where the change in composition of the plaque flora, from two hours to three weeks, was investigated. A study of the microbial succession in developing plaque has obvious relevance when investigating the relationship between plaque microflora and initiation of enamel demineralisation, since it is important to know the changes in proportions and absolute counts of specific organisms throughout the duration of the study, rather than simply at the end of the experimental period, as was the case in the main appliance study.

The findings that the application of sucrose to one side of the appliance did not affect the plaque pH on the opposite side (as shown in Section 3.4.5.), and that the implantation of Strep. mutans on one side did not increase the counts of this organism on the other side (see Section 4.6.7.), indicate that the appliance model can be used to investigate the effects of different treatment conditions on plaque microflora and enamel demineralisation, with one side of the appliance acting as a control.

Therefore, the current studies were designed to utilise the advantages of the appliance model, as discussed above, and the results of these investigations into plaque microflora and associated enamel demineralisation are discussed in subsequent Sections.

7.5.2. Streptococcus mutans and Demineralisation.

Numerous in vivo studies have implicated Strep. mutans in the aetiology of the initiation of enamel demineralisation. The results of the appliance study, described in Chapter IV, found a positive relationship between Strep. mutans and enamel demineralisation with regard to isolation frequency, mean and median proportional counts of this organism. The findings of high Strep. mutans levels in association with sound enamel, and a few cases of mineral loss of between 500 and 1,000 Δz units, in the apparent absence of Strep. mutans in overlying plaque, are also similar to the in vivo results of other workers. It is possible that the organisms were present, but undetected, in the plaque overlying demineralised sites, due to the presence of microcolonies of Strep. mutans being diluted in the

plaque sample so as to become a negligible part of the total flora. However, this is more of a problem in in vivo studies, where accurate plaque sampling from the caries-prone sites is difficult, while the appliance study involved small discrete enamel sites from which accurate plaque sampling could be achieved. Thus, although the experimental design reduced the problem of microcolonies being diluted within a plaque sample, it did not entirely eliminate this possibility. However, it may have been that Strep. mutans was indeed absent from a few plaque samples associated with mineral loss, suggesting that other acidogenic organisms may be capable of initiating demineralisation. This would also be a possibility in the many sites where demineralisation occurred, but Strep. mutans comprised 0.1 % or less of the total microflora.

It has been shown in longitudinal studies that a rise in Strep. mutans occurs in plaque prior to detection of a lesion in the associated enamel. Therefore, the absence of demineralisation despite the presence of Strep. mutans in high proportion in the current study, may have been due to the short experimental period employed. Thus, had the duration of the experiment been extended, demineralisation may have occurred at these sites. The spatial arrangement of bacteria within plaque may also be relevant in determining whether or not mineral loss from the enamel surface will take place, and may help to explain the presence of large numbers of Strep. mutans in the absence of underlying demineralisation.

Another possible explanation for the lack of correlation between Strep. mutans counts and mineral loss in associated enamel, is the varying cariogenic potential of different strains of the organism. The study reported in Chapter V, involving one strain of Strep. mutans from each volunteer, showed a significant correlation between in vitro cariogenic potential and the level of in situ demineralisation asociated with the different strains.

A further advantage of the appliance model was that, in addition to the use of sucrose stressing of the plaque as described in Section 7.4., in some experimental runs, inoculation of the subject's own Strep. mutans on to the test sites could be performed. The runs involving implantation of Strep. mutans and sucrose stressing resulted in increased levels of demineralisation, overall, compared to those associated with normal and

sucrose plaques. However, considerable inter-subject variation occurred following implantation of Strep. mutans, for, although the isolation frequency of the organism increased for all subjects, the mean proportion of Strep. mutans was little changed in some individuals. This is similar to the results of other workers, who found variable implantation success in different volunteers.

The possible reasons for the varying success of implantation have been discussed in Chapter IV, and the study described in Chapter VI, which monitored the proportional and absolute counts of Strep. mutans in early plaque, following implantation of this organism, suggested that a threshold count may exist, above which the organism will become a predominant member of the plaque microflora when exposed to regular sucrose applications.

Only one of the subjects involved in the appliance study had high natural counts of Strep. mutans in plaque, and this subject showed high levels of demineralisation under normal and sucrose plaque conditions. In the others, little demineralisation was seen under these protocols, whilst, overall, increased mineral loss was noted following Strep. mutans implantation. Therefore, selection of individuals with higher salivary and plaque levels of this organism might have resulted in greater amounts of demineralisation under normal and sucrose plaque conditions, and not necessitated the implantation of Strep. mutans on to test sites.

7.5.3. Lactobacillus spp. and Demineralisation.

The in situ demineralisation study found a positive correlation between plaque lactobacillus levels and enamel demineralisation of over 1,000 units. This result is in agreement with those of other workers in supporting the view that this organism is associated with established lesions.

The isolation frequency of Lactobacillus in plaque associated with sound sites was higher than that generally reported in clinical studies. This may have been due to the increased retentiveness of the appliance test sites, compared to the natural dentition. Thus, as Lactobacillus was frequently present, although in low proportion, in plaque associated with

smaller enamel lesions, its role in the initiation of enamel demineralisation remains unclear.

7.5.4. The Role of Other Organisms in Demineralisation.

In some of the sites where mineral loss was between 500 - 1,000 Δz units, neither Strep. mutans nor Lactobacillus spp. was isolated from related plaque. This suggests that other acidogenic organisms may have been responsible for the demineralisation in these cases. However, no relationship was found between either isolation frequency or mean count and increasing demineralisation, for any of the other organisms identified. The slight fall in mean proportion of Strep. sanguis and Strep. oralis, with increasing mineral loss, is in keeping with the findings of other workers, and no trend was evident with regard to Actinomyces. However, these streptococci and Actinomyces spp. comprised the majority of organisms isolated from the supra-gingival plaque samples, with a change in dominance from streptococci to Actinomyces occurring between 7 and 21 days, as described in Section 6.4.3. Therefore, these organisms merit further investigation, and as Strep. mutans comprised only a small proportion of the total flora, yet showed a significant correlation between in vitro cariogenic potential and in situ demineralisation, it would be interesting to perform similar in vitro tests on Strep. sanguis, Strep. oralis and Actinomyces spp. In addition, the cariogenic potential of lactobacilli should be investigated, thus determining whether any correlation exists between the in vitro cariogenic potential of any of these organisms and the natural and experimental caries experience of the subjects from whom the strains were isolated.

Furthermore, Strep. mutans and lactobacilli were frequently isolated only on selective media, while the non-selective plates were the only medium available for isolation of other plaque organisms. Therefore, a positive correlation between isolation frequency and demineralisation in humans cannot be excluded for such organisms unless studies with appropriate selective media are undertaken, although animal and in vitro investigations would suggest that these other organisms are not so strongly implicated in the carious process.

7.5.5. Summary of Relationship Between Plaque Microflora and Enamel Demineralisation.

The fact that there was a rise in isolation frequency, mean and median counts of Strep. mutans in plaque samples associated with increasing demineralisation, suggests that this organism is strongly implicated in the initiation and development of caries, and is in agreement with findings reported from clinical studies. The proportion of lactobacilli was significantly higher in plaque samples associated with only the most extensive enamel lesions, supporting the view that this organism is selected for by the acidic conditions associated with early demineralisation, and may then be involved in the progression of the lesion.

However, the results of the present studies suggest that, on some occasions, lesions may have developed in the absence of both Strep. mutans and Lactobacillus spp. Thus other acidogenic organisms present in plaque may, either singly or in combination, have been capable of initiating enamel demineralisation.

Unfortunately, the multifactorial nature of the disease process makes it impossible to relate categorically the plaque microflora to demineralisation in the human oral cavity, although the use of the in situ appliance enabled attempts at standardisation of experimental conditions to be made.

Furthermore, while initial studies showed that factors such as experimental run, and subject, influenced the results to some extent, particularly because of the small number of volunteers involved, the statistical package chosen for the analysis of the results took these factors into account.

7.6. Assessment of Cariogenic Risk.

In addition to attempting to standardise experimental conditions by using the intra-oral appliance, as discussed in 7.5.5., other factors such as salivary microbial counts, salivary buffering capacity and flow rate, and diet, which might influence the carious process, were also studied to try to obtain as comprehensive a picture as possible of the inter-relationship of the variables involved in each subject's mouth. This has only very rarely been performed in other microbiological caries studies. While there

was a trend towards a relationship between salivary counts of Strep. mutans and lactobacilli and in situ demineralisation, this did not reach a significant level, and no correlation between frequency of intake of fermentable carbohydrate and mineral loss was found, as discussed in Section 7.4. In addition to these commonly used caries predictive tests, in vitro testing of the cariogenic potential of a Strep. mutans strain from each subject was performed. Differences were found in the acidogenic and demineralising potential among the strains, and the cariogenic potential of the Strep. mutans strains was found to correlate with the DMFS score and in situ demineralisation levels of the subjects from whom the strains were isolated. While this was only a preliminary study, involving one strain per volunteer, the results appear to support the concept that, in addition to actual numbers or proportions of specific bacteria in saliva or plaque, the pathogenicity of individual organisms may vary, and this should also be considered when assessing caries risk.

This study was unique in allowing the cariogenic potential of the Strep. mutans strains to be compared with both the subject's past caries experience and very recent short-term experimental demineralisation scores. If a child population were to be used in a future longitudinal caries study, this would allow a correlation between past caries experience and caries increment during the study, and the cariogenic potential of the children's Strep. mutans strains to be made.

7.7. Conclusions and Suggestions for Future Studies.

Chapter IV describes an investigation of the plaque flora composition associated with enamel demineralisation, using a method which has not previously been employed. The use of thin enamel sections, discrete and readily accessible test sites for plaque sampling, and the ability to stress the test areas to allow rapid demineralisation to be produced without affecting the volunteers' natural dentition, overcame many problems associated with in vivo cross-sectional and longitudinal studies.

Overall, the results showed an association between Strep. mutans and mineral loss, and found high levels of lactobacilli associated with the more extensive lesions. However, as has been found in most other studies, the relationship between Strep. mutans and demineralisation varied between

sites, suggesting that other plaque organisms also play an important role in the carious process, either by creating an acidic environment capable of producing mineral loss in the absence of Strep. mutans, or by modifying the challenge of this organism. This could be achieved either by metabolising the acid formed, or possibly by non-acidogenic organisms being positioned within plaque between the Strep. mutans cells and the enamel surface.

While the multifactorial nature of this disease means that it is impossible to relate directly plaque microflora and demineralisation in the human oral cavity due to the inability to exclude co-existing factors, the use of this model can control or monitor some of these influences. It is therefore a useful tool in the investigation of the microbiology of dental caries initiation, and the results obtained suggest that further work, using the appliance, possibly with some modifications as outlined in the preceeding sections, should be undertaken.

The appliance also has potential for use in early plaque development studies, since multiple enamel slabs mounted on each side of the device offer a model which allows i] longitudinal studies with plaque sampling at different times to be performed, ii] isolates to be expressed in terms of proportional and absolute counts, and iii] different treatment conditions to be tested simultaneously under the same general intra-oral conditions. Thus, further development of this model, for use on larger subject samples seems indicated, and its possible use in investigations of the effect of antimicrobial agents on plaque microflora could be explored.

Finally, results obtained from the in vitro study described in Chapter V suggest that the differences which exist in cariogenic potential of individual strains of Strep. mutans may help to explain why a relationship between the numbers or proportions of Strep. mutans in a plaque sample and the caries status of the associated enamel site, is not always seen. A significant correlation between the cariogenic potential of the strains, and the natural and experimental caries experience of the individuals from whom they were isolated, was found. This significant relationship was observed with three of the four parameters used to assess cariogenic potential, namely the total acid anion concentration, minimum pH level reached, and calcium released from the enamel slabs. In the present

study, samples were removed for acid anion analysis on six occasions during every 24 hour period. In addition to repeating this study using a larger subject number and multiple strains of Strep. mutans from the same individual, attempts could be made to streamline the model by reducing the number of sampling occasions, and determining whether all four methods of assessing cariogenic potential are necessary. In this way, an indication could be obtained as to whether this model has potential for development as an additional screening test to aid in the detection of high caries risk individuals.

APPENDIX I.

Derivation of the Equation by Angmar and co-workers (1963).

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

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

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

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

and $V_m/V_s = t_m/t_s$

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

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

The absorption coefficients depend on the radiation source (eg. kV, target, filter, etc.). Angmar and co-workers (1963) employed CuKα radiation; μ_a, μ_m and μ_o were found from known data, and thus the equation reduces to,

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

Thus, the only unknown is t_a, since t_s can be measured (see Section

2.3.4.). Therefore, for every point in the enamel, the equivalent aluminium thickness (t_a) is derived and percent volume mineral calculated.

APPENDIX II.

Preparation of Media.

Pouring of Plates.

All plates were poured in a clean air laminar flow cabinet (Microflow Pathfinder, Intermed). Each plate contained approximately 15 ml of medium, and was allowed to cool and surface dry for 20 minutes before its lid was applied. All plates were stored at 4°C before use, and were used within one week of preparation.

Media :

Anaerobic Blood Broth.
Chemically Defined Medium for Streptococcus mutans.
Mitis Salivarius Bacitracin Agar.
Rogosa S L Agar.
Todd - Hewitt Broth.
Tryptic Soy Blood Agar.

Anaerobic Blood Broth.

Source : Gibco - Europe Ltd., Paisley, Scotland.

Composition :

	grams per litre
Peptone 140	10.0
Beef extract	2.0
Liver digest	3.0
Yeast extract	5.0
Sodium chloride	5.0
Dextrose	5.0
Haemin	0.005
Menadione	0.005
L - cysteine	1.0
Dithiothreitol	0.1
Sodium bicarbonate	0.9

Method of Preparation.

Thirty two grams of powder were added to one litre of distilled water. After thorough mixing and checking that the pH was 7.3, 20 ml volumes of the medium were dispensed into universal containers. These were then capped tightly and autoclaved at 121°C for 15 minutes.

Chemically Defined Medium for Streptococcus mutans.
Terleckyi, Willett and Shockman (1975)

Composition :

	grams per litre
Sodium acetate	6.0
Ammonium sulphate	0.6
Adenine	0.035
Guanine	0.027
Uracil	0.03
Manganese sulphate	0.2
Sodium chloride	0.01
Ferrous sulphate	0.01
MnSO	0.01
Glutamine	0.005
L - glutamic acid	0.3
L - lysine	0.11
L - aspartate	0.1
L - isoleucine	0.1
L - leucine	0.1
L - methionine	0.1
L - serine	0.1
L - phenylalanine	0.1
L - threonine	0.1
L - valine	0.1
DL - alanine	0.2
L - arginine	0.2
L - cystine	0.2
L - histidine	0.2
Glycine	0.2
L - hydroxyproline	0.2
L - proline	0.2
L - tryptophan	0.2
L - tyrosine	0.2
Sodium hydrogenphosphate	3.15
Sodium dihydrogenphosphate	2.05

Potassium hydrogenphosphate	0.3
Potassium dihydrogenphosphate	0.44
Sodium citrate	0.225
Riboflavin	0.0004
p-aminobenzoic acid	0.0001
Thiamine	0.0004
Nicotinamide	0.002
Pyridoxamine	0.0008

Method of Preparation.

The above constituents were added to one litre of distilled water, and the medium mixed overnight using a magnetic stirrer. Glucose was then added at a concentration of 20 grams per litre, and the pH adjusted to pH 6.5 ± 0.1 with NaOH. The medium was then filter-sterilised through a 0.45 μm membrane filter and dispensed aseptically into 20 ml sterile bottles and stored at 4° C.

Mitis Salivarius Bacitracin Agar (MSB).
Gold, Jordan and van Houte (1973).

Source : Difco Laboratories, East Malling, Surrey, England.

Composition (of Mitis Salivarius Agar) :

	grams per litre.
Bacto - Tryptase	10.0
Proteose Peptone No 3, Difco	5.0
Proteose Peptone, Difco	5.0
Bacto - Dextrose	1.0
Saccharose, Difco	50.0
Dipotassium phosphate	4.0
Trypan Blue	0.075
Bacto - Crystal Violet	0.0008
Bacto - Agar	15.0

Method of Preparation.

Ninety grams of Mitis Salivarius Agar, and 150 g of additional sucrose were added to one litre of distilled water and dissolved in a Koch steamer. After checking that the pH was 7.0, the medium was dispensed into bottles, in 100 ml and 500 ml volumes, and autoclaved at 121 °C for 15 minutes. When the medium was required, the agar was melted in a Koch steamer. It was then cooled to 45 °C, and 1.0 ml of 1 % Bacto Chapman Tellurite (Difco Laboratories Ltd.) and 10 ml of a sterile solution, containing 20 units of bacitracin (Sigma Chemical Co.) per ml were added to each litre of medium. Plates were then poured.

Rogosa SL Agar.

Rogosa, Mitchell and Wiseman (1951).

Source : Difco Laboratories, East Malling, Surrey, England.

Composition :

	grams per litre.
Bacto - tryptone	10.0
Bacto - Yeast Extract	5.0
Bacto - Arabinose	5.0
Bacto - Saccharose	5.0
Sodium Acetate	15.0
Ammonium Citrate	2.0
Monopotassium phosphate	6.0
Magnesium sulphate	0.57
Manganese Sulphate	0.12
Ferrous Sulphate	0.03
Sorbitan monooleate	1.0
Bacto - Agar	15.0

Method of Preparation.

Seventy five grams of powder were added to one litre of distilled water and dissolved in a Koch steamer. For each litre of medium, 1.32 ml of glacial acetic acid (BDH Chemicals Ltd.) was added. After checking that the pH was 5.4, the medium was boiled for a further 2 - 3 minutes. It was then allowed to cool and plates were poured.

Todd - Hewitt Broth.

Todd and Hewitt (1932)

Source : Gibco - Europe Ltd, Paisley, Scotland.

Composition :

	grams per litre
Beef Heart Infusion	3.1
Peptone 180	20.0
Dextrose	2.0
Sodium Chloride	2.0
Sodium Phosphate Dibasic	0.4
Sodium Carbonate	2.5

Method of Preparation.

Thirty grams of powder were added to one litre of distilled water, and dissolved in a Koch steamer. After checking that the pH was 7.8, 20 ml volumes of the medium were dispensed into universal containers. These were then capped tightly and autoclaved at 121 °C for 15 minutes.

Tryptic Soy Blood Agar.

Source : Gibco - Europe Ltd. Paisley, Scotland.

Composition (of Tryptic Soy Agar) :

	grams per litre
Peptone 140	15.0
Peptone 110	5.0
Sodium Chloride	5.0
Agar	15.0

Method of Preparation.

Forty grams of powder were dissolved in one litre of distilled water in a Koch steamer. After checking that the pH was 7.3, the medium was autoclaved at 121 °C for 15 minutes. The medium was then allowed to cool to 50 °C, and 7.5 % defibrinated horse blood and 1 % Vitamin K / Haemin added. Plates were then poured.

APPENDIX III.

Demineralising Solution for *in vitro* Enamel Susceptibility Study.
Damato, Strang and Stephen (1988).

Composition :

Calcium chloride	0.2219 g / litre
Sodium hydrogen phosphate	0.3120 g / litre
glacial acetic acid	2.875 ml / litre

Method of preparation.

The $CaCl_2$ and NaH_2PO_4 were weighed and added to approximately 800 ml of double-distilled deionised water. Glacial acetic acid (2.875 ml) was then added and the pH adjusted to 4.8 with NaOH. The solution was then made up to one litre by the addition of double distilled deionised water.

APPENDIX IV.

INFORMATION SHEET FOR VOLUNTEERS.

"Microbial Factors Involved in the Early De- and Remineralisation of Dental Hard Tissues".

The purpose of this study is to characterise accurately the microorganisms and environmental conditions necessary for the early demineralisation of enamel to take place.

The involvement of volunteers in the study is as follows :

1. Screening Tests.
   Before inclusion in the study it will be necessary for a number of screening tests to be carried out :
   a] Intra-oral examination by dentist to record past and current caries activity.
   b] Collection of a 5 ml mixed salivary sample, stimulated by chewing a paraffin wax pellet.
   c] Dental plaque samples collected from two lower teeth.

2. Construction of Intra-oral Appliance.
   If after the above screening test the volunteer is accepted into the full study then it will be necessary to take a full upper and lower alginate impression in order to construct a lower acrylic appliance.

3. Experimental Procedures.
   On to the lower appliance will be attached very small sections or pieces of enamel, which have been thoroughly cleaned and sterilised. The appliance will be worn for periods of up to 3 weeks maximum. Volunteers will be instructed to remove the appliance from the mouth to carry out the following procedures :
   a] Normal toothbrushing twice daily using a non-fluoridated paste.
   b] Flossing once daily.
   c] General cleaning of the appliance twice daily, but leaving the test area untouched.
   d] Sucrose applications - a 10 % sucrose solution will be applied

extra-orally to the test area of the appliance for periods of one minute. This will be carried out nine times daily.

e] In addition to the above procedure it may be necessary to inoculate the test area on the appliance with the **volunteer's own Strep. mutans** in order to produce measurable demineralisation on the test enamel sections.

4. Safety.

Theoretically there is no reason to expect that as a result of wearing the appliance as described previously, volunteers would experience a sudden or long term increase in dental disease or any other health problems. One of the researchers has personally worn the appliance under experimental conditions for over one year without any detectable change in dental or general health.

GREATER GLASGOW HEALTH BOARD.

Glasgow Dental Hospital and School.

Consent Form.

SURNAME : .....

FORENAMES : .....

I ..... of .....

hereby consent to take part in the study on "Microbial Factors in the Early De- and Remineralisation of Dental Hard Tissues", the nature and effect of which have been explained to me by :

Researcher : .....

I understand that I can withdraw from the study at any time.

Date : ..... Signed : .....

I confirm that I have explained the nature and effect of participation in this research study to the volunteer.

Date : ..... Signed : .....

Researcher's Signature.

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