IN VITRO TESTS TO DETERMINE THE CARIOGENIC POTENTIAL OF ORAL MICROORGANISMS.

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This thesis is the original work of the author.

SUMMARY

In the aetiology of dental caries, a definite bacterial role was first proposed by W.D. Miller (1890). In the interim, many researchers have investigated the relationship of oral microorganisms to the disease process. Since the 1960's, members of the mutans streptococci have been implicated consistently as playing a major role in dental caries, due mainly to significant correlations between the number of these streptococci in saliva, and caries experience.

The recent decline in caries prevalence has demonstrated that individuals differ in caries susceptibility, and within communities there exist high-risk groups and individuals. Such findings have highlighted the desirability of determining accurately the caries susceptibility of an individual patient prior to, or at a very early stage of disease initiation. Currently available caries-activity tests have concentrated mainly on the numbers of organisms present in saliva, with no account taken of potential differences in virulence. Increasingly, it has been realised that such tests lack sufficient sensitivity and specificity to permit reliable identification of the at-risk individual, rendering them of limited usefulness clinically.

Macpherson (1988), suggested that strains of S. mutans differ in their cariogenic potential and that such differences correlated with clinical disease experience. This thesis aimed to investigate further, the in vitro cariogenic potential of oral microorganisms.

The microbiological methods and analytical techniques used in the study, and the isolation, identification and storage of clinical isolates were described (Chapter 2). The technique of isotachophoresis was also detailed, and previously unreported factors influencing

isotachophoretic analysis were identified whilst validating the technique. This Chapter concluded by describing the detection of calcium by complexation with o-cresolphthalein and compared the technique with atomic adsorption spectroscopy.

The *in vitro* test system described by Macpherson (1988), involved the incubation of a bacterial cell slurry, a slab of bovine enamel, and a 5% sucrose solution for two, 24 hour incubation periods at 37°C. Acid production, calcium release and changes in pH were assayed as determinants of *in vitro* cariogenic potential. In initial studies, five of the six strains studied by Macpherson (1988), were investigated (Chapter 3). Hydroxyapatite in both a solid and powdered form were used as alternatives to bovine enamel. The findings of these investigations were in broad agreement with previous results of the *in vitro* cariogenic potential of the strains and, furthermore, demonstrated that by substituting powdered hydroxyapatite for bovine enamel, the incubation period could be reduced to five hours. It was decided to use the latter model for the remainder of the study.

The *in vitro* cariogenic potential of strains representing six streptococcal species was investigated next (Chapter 4). Results indicated that the species could be ranked into three homogeneous groups on the basis of acid production, calcium release and pH after five hours' incubation, with *E. faecalis*, *S. gordonii*, and *S. sanguis* of low; *S. vestibularis*, of intermediate; and *S. mutans* and *S. sobrinus* of high cariogenic potential. These findings were in agreement with previous determinations of the relative pathogenicity of these species in animal models.

In Chapter 5, a study to investigate the relationship

between the *in vitro* cariogenic potential of dental plaque isolated from 51 teenagers, and their clinical caries experience is described. Whilst significant relationships were observed between the determinants of *in vitro* cariogenic potential, it was not possible to demonstrate significant relationships with clinical disease experience.

The remaining studies described in Chapters 6, 7 and 8 concentrated on the investigation of the cariogenic potential of *Streptococcus mutans* strains isolated from children participating in a clinical trial.

In Chapter 6, significant relationships were observed between salivary counts of mutans streptococci and caries experience in 60 twelve-year-olds. Within this group there existed children who, in spite of having high counts of mutans streptococci remained clinically caries-free and, conversely, children with high caries scores but relatively low counts of mutans streptococci. Strains of *S. mutans* isolated from twenty children, nine of whom were clinically caries-free and eleven with high disease experience, were then tested in the *in vitro* model. Whilst higher mean acid production and calcium release were observed with strains isolated from the high-caries group, the values obtained were not significantly different from those obtained with strains isolated from the caries-free children. In the final experiment described in Chapter 6, five strains of diverse *in vitro* cariogenic potential were investigated further, and significant differences in their cariogenicity demonstrated.

It was decided that if a large number of strains were to be tested on a longitudinal basis, variability on repeated testing must be low, in order that small variations in cariogenic potential could be demonstrated.

The variability in the cariogenic potential of strains isolated from different individuals, of strains isolated from one individual, and variation on repeated testing were investigated (Chapter 7). Results showed that the variability on repeat testing was such that reliable differentiation between strains of S. mutans, on a longitudinal basis, was not possible. Potential factors influencing variability were therefore examined (Chapter 8) and the relationship of in vitro cariogenic potential determinants during the incubation investigated (Section 8.1). It was concluded that, whilst the fall in pH was greatest in the initial period, acid production and calcium release were influenced by bacterial metabolism throughout the incubation. Next, the effect of the individual components of the in vitro model on cariogenic potential was investigated (Section 8.2). Whilst the metabolism of the bacterial cells was shown to be influenced significantly by the presence of hydroxyapatite, no factors which could have accounted satisfactorily for the variation observed in the experiments described in Chapter 7 were identified.

In conclusion, whilst the model has been used to discriminate between species of streptococci, with the exception of a few selected strains, it was not possible to discriminate reliably between the majority of wild type S. mutans strains. It seems unlikely that further investigation of the virulence of mutans streptococci strains, using the experimental approach described in this thesis, would contribute significantly to improving the sensitivity and specificity of microbiological caries-activity tests.

ABBREVIATIONS

°C = Degrees centigrade.

c.f.u. = colony forming units.

Ch. = Chapter.

cm. = centimetre.

d.f. = degrees of freedom.

dl. = decilitre.

ed. = editor or edition.

e.g. = exempli gratia, for instance.

etc. = etcetera.

g. = gravity.

h. = hours.

i.e. = id est, that is.

LD_{50} = Lethal Dose 50

mbar. = millibar.

mg. = milligram.

min. = minute.

ml. = millilitre

mm. = millimetre.

mM. = millimolar.

M.S.B. = Mitis Salivarius Bacitracin.

n. = number.

N.D. = not determined.

nmol. = nanomoles.

No. = Number.

N.S. = not significant.

pp. = pages.

P.B.S. = Phosphate Buffered Saline (pH 7.2).

rpm = revolutions per minute.

List of abbreviations continued.

S.D. = Standard Deviation.

S.L. = Selective for Lactobacilli.

S.M. = Streptococcus mutans.

spp. = species.

supp. = supplement.

T.H.B. = Todd Hewitt Broth.

T.Y.C.S.B. = Trypticase Yeast Cystine Sucrose Bacitracin.

µl. = microlitres

vol. = volume.

vs. = versus.

W. = Watt.

wt. = weight.

w/v. = weight/volume.

Whilst the name Streptococcus faecalis has been used in this thesis, it is now generally accepted that this species is more appropriately termed Enterococcus faecalis.

CHAPTER 1

INTRODUCTION, LITERATURE REVIEW, AND AIMS.

1.1 Introduction.

Dental caries is a complex chronic disease, and the principal aetiological factors have been relatively clearly defined since Miller first proposed the chemico-parasitic theory in 1890. In the interim, a vast body of knowledge relating to the pathogenesis, prevention, and treatment of the disease has been published (Bratthall 1992).

However, in the year in which the study described in this thesis was begun, £934, 600, 000 was spent in the United Kingdom on General Dental Services within the National Health Service, mainly in treating caries (Seward, 1990). In the same period, 410,000 general anaesthetics were given within National Health Service general dental practice, some 250,000 of which were administered to children under fifteen years of age. These figures are surprisingly high for a country in which dental caries has been declining for at least fifteen years (Renson, 1985). It is apparent that despite considerable advances in caries research, not all individuals have benefitted equally from the decline in caries prevalence in industrialised countries. Furthermore, in many developing countries, the prevalence of dental caries is increasing (Murray, 1986).

The ability to identify individuals who are susceptible to a specific disease is a highly desirable aim in disease prevention, and has long been a goal of workers in the field of cariology (Koch, 1988). However, despite many optimistic reports, few truly useful, practical tests are available to the dental team, for routine deployment in the clinic to determine the caries risk of an *individual* patient, and this deficiency formed the basis of the study described in this thesis.

1.2 The aetiology of dental caries.

In simple terms, the chemoparasitic theory of Miller states that bacteria in the mouth react with food particles to produce acid capable of dissolving tooth enamel.

In 1962, the principal aetiological features of caries were summarised by Keyes who published a diagram which consists of three overlapping circles depicting host, dietary and microbial factors. This now ubiquitous diagram indicates that, for dental caries to occur, all three parameters are required to be present concurrently, over an unidentified time period. However, a large number of local and general predisposing factors, whose exact role in the disease aetiology is unclear, have also been identified.

Johnson (1991), has stated that dental caries is a process with no dramatic or easily identified starting or

end point and is, in fact, a complex, dynamic and continuous biological process which cannot be defined in terms of a single event, observation, or substance. This author discussed in detail the multiplicity of factors that may influence the initiation, rate of progression or regression of the caries process.

The following Sections review briefly the role of host and dietary factors in caries aetiology, before a more detailed discussion of the role of microorganisms in dental caries.

1.2.1 Host factors in caries aetiology.

Host factors involved in caries aetiology can be considered as local (i.e. those operating within an individual mouth), and general.

Of the local factors, the form and arrangement of the teeth (including the number of teeth), fissure patterns and occlusion have all been investigated as determinants of caries risk (Hunter, 1988). Other local factors of importance are oral hygiene status (Sutcliffe, 1989), and salivary properties (Pearce, 1991; Tenovuo, Lumikari & Soukka, 1991). The general factors that have been investigated include age, sex, race, geographical location and social factors. However, to determine accurately the contribution of each of these parameters to dental decay is extremely difficult, as caries development is influenced by the whole socio-cultural

environment of the community in which an individual resides (Hunter, 1988).

1.2.2 Dietary factors in caries aetiology.

Diet can affect the teeth in two ways, namely whilst the tooth is forming, i.e the pre-eruptive effect, and a local oral effect after the tooth has erupted into the oral cavity (Rugg-Gunn, 1989). The influence of diet on caries has been reported widely, evidence coming from animal studies, and from human observational and interventional works. Sreenby (1982) concluded that the formation of caries was significantly influenced by both the total amount of sugar consumed and the frequency of intake. Furthermore, it is likely that the length of time that teeth are exposed to sugar containing foods, rather than simply the form of the food, is the critical factor in the promotion of caries. Hunter (1988) reported that whilst many questions remain to be answered about the relationship between diet and caries, diet is a dominant variable in determining caries prevalence in a community. Holm (1990), has recently commented on the difficulties encountered in accurately recording dietary data for individual patients.

1.2.3 Microbial factors in caries aetiology.

The third major variable involved in the aetiology of dental caries is the microflora, the role of which is discussed in Sections 1.3 - 1.12.

1.3 The oral flora.

The mouth, in common with all environmentally exposed areas of the human body, harbours a complex microbial flora (Marsh 1991) which, it has been estimated, affords residence to some 10^{10} bacterial cells (Williams, 1973), comprising at least thirty bacterial genera (Hardie, 1992), or 300 different bacterial species (Moore et al., 1982).

At birth the mouth is generally sterile (Marsh 1991), but is colonised within a few hours post partum, (McCarthy, Snyder & Parker 1965). A number of authors have reported on the chronology and the sequence of appearance of different types of bacteria (Socransky & Manganiello 1971), as well as on the source, route of transmission, and preferred intra-oral habitats of specific bacterial species (Jordan, 1976). It is, however, apparent that relatively few such studies have been reported in recent years, a surprising finding in light of the many changes in bacterial taxonomy over the past twenty years.

A diverse range of potential habitats exists within the oral cavity, which vary with respect to surface characteristics, oxygen levels and anaerobiosis, availability of nutrients, exposure to salivary secretions or gingival crevicular fluid, masticatory forces and other variables such as oral hygiene

procedures (Hardie, 1992). Bacteria indigenous to the human mouth vary considerably in their affinity for different oral surfaces, and the composition of the microbial flora also varies from site to site, reflecting both physical and environmental parameters.

Teeth provide "non-shedding" surfaces so that, in the absence of oral hygiene practices, large accumulations of microorganisms and their products develop in stagnant or retentive sites, and are termed dental plaque as reviewed by Marsh, (1991). The presence of plaque is a necessary prerequisite for the development of dental caries (Löe, 1969).

1.4 Evidence of bacterial involvement in dental caries.

Evidence for the intrinsic role of bacteria in the caries process was provided by McClure and Hewitt (1946), who demonstrated that the addition of penicillin to the diet of rats prevented caries. However, proof of the central role of microorganisms in the aetiology of dental caries, is generally attributed to the classical germ-free animal studies of Orland et al. (1954, 1955).

Further evidence of the integral role of bacteria in caries aetiology comes from a number of sources. In vitro studies have demonstrated that caries-like lesions can be created when enamel slabs are incubated with bacterial slurries (Sperber & Bunocore, 1963; Gray 1966; Featherstone & Rodgers, 1981; Gallagher, Pearce &

Cutress, 1983), and microorganisms have been demonstrated invading carious enamel and dentine (Soames & Southam, 1985; Hahn, Falker & Minah, 1991; McCabe, Adamkiewicz & Pekovic, 1991), from which they can be isolated and cultured (Shovelton, 1968). Furthermore, clinical experience confirms the observation of Baab, Morton and Page (1984), who reported that teeth which are not exposed to the oral environment (and thus are not significantly exposed to bacteria) do not develop caries. However, whilst it is now agreed that the disease cannot occur in the absence of bacteria, there remain differences of opinion as to the role of microorganisms in the production of carious lesions (Newbrun, 1989).

1.5 Factors influencing the cariogenic potential of oral microorganisms.

Before discussing further the evidence implicating specific bacterial species in the caries process, this Section reviews factors which may influence the cariogenic potential of oral microorganisms.

Virulence is a measure of an organism's pathogenic potential (Berry, Peterson & Davis 1986), or ability to cause disease. In medical microbiology, factors known to affect virulence include toxin production, the presence of capsules, and the ability to adhere to body surfaces. Experimental measures of virulence can be obtained both in vitro and in vivo in animal models, the best known

example being the LD_{50} test which determines the dose of organisms required to kill fifty percent of a study population (Shanson, 1982). It is, however, much more difficult to precisely determine the cariogenicity of oral microorganisms.

1.5.1 Physiological traits enhancing the cariogenic potential of oral microorganisms.

Whilst it is not known what exactly determines the cariogenic potential of a bacterial cell (Newbrun 1989), several phenotypic traits (investigated mainly in mutans streptococci), have been shown to be of importance in determining virulence. These include the ability to adhere to tooth surfaces, to aggregate with other bacteria in dental plaque, to transport sucrose and other sugars by energy-dependant processes, to be acidogenic from a variety of simple sugars especially sucrose, to manufacture lactic acid, to be aciduric, and to form glycogen-like intracellular polysaccharides (Tanzer, 1989).

The relative importance of several of these traits has been determined by comparing the virulence of mutants of mutans streptococci having specific, limited phenotypic defects, with wild-type progenitors in experimental animal models. Mutants defective in both the synthesis of insoluble glucans (De Stoppelar et al., 1971; Tanzer et al., 1974) and in the formation of intracellular

polysaccharides (Tanzer et al., 1976) have shown reduced smooth surface caries activity in rodent models. A similar result affecting both smooth surface and fissure caries was obtained with a mutant defective in lactate dehydrogenase activity (Johnson, Gross & Hillman, 1980).

It has been suggested (Williams, 1973; Tanzer, 1989), that a potential means of influencing the cariogenic potential of dental plaque would be the replacement of cariogenic organisms by mutants of lesser or negligible cariogenic potential. Such a strategy would, however, be dependant on the avirulent strain successfully competing with the indigenous flora at the expense of all potentially cariogenic organisms, and would probably require inoculation of such an organism at birth.

Recently, Kitamura et al., (1989) investigated a bacteriocin-producing strain of S. sobrinus and suggested that such a property permitted this strain to multiply at the expense of S. mutans, when grown in specific pathogen-free rats. It is therefore possible that the presence of bacteriocinogenic bacteria may alter the cariogenic potential of dental plaque by disrupting the plaque ecosystem.

1.5.2 Physiological traits reducing the cariogenic potential of oral microorganisms.

Members of the commensal flora may possess phenotypic

traits which act to create a less cariogenic environment, either directly as a result of their own metabolic characteristics, or by influencing those of other bacterial species. As reviewed Edwardsson (1986), interest has been focused on the contribution of so-called "pH rise" factors. The production of alkali results principally from the metabolism of urea to ammonia and carbon dioxide, and by the decarboxylation of plaque amino acids to amines. However, the extent to which alkali production by plaque bacteria influences and modifies the initiation of caries is not known. The utilisation of acid end products, principally lactate, to weaker acids has also been proposed as a pH-regulating factor. Mikx et al. (1972), suggested that the cariogenic potential of lactate-producing organisms may be reduced by the lactate utilising effect of members of the genus Veillonella.

1.5.3 Influence of host defences on the cariogenic potential of oral microorganisms.

The mouth is provided with both specific and non-specific host defences (Marsh, 1989). Many diseases of bacterial origin have been prevented by mobilisation or augmentation of the defence systems of the body, and the idea that the cariogenic potential of oral microorganisms may be modified by such methods has a long history. However, despite intensive research, it would appear that a clinically useful caries vaccine remains an unrealised

objective (Edgar, 1989). Interestingly, Tenovuo et al. (1991), have suggested utilising the non-specific defense mechanisms of saliva to influence the cariogenic potential of oral microorganisms by adding antimicrobial proteins such as peroxides, lactoferrin and lysozyme to oral health products.

1.5.4 Influence of salivary factors on the cariogenic potential of oral microorganisms.

Teeth are bathed in saliva, and this medium may therefore influence the cariogenic potential of bacteria in a number of ways. Physical properties of saliva, such as viscosity, spinbarkeit and flow-rate, may influence bacterial colonisation and exert a physical washing effect, in addition to clearing potential bacterial nutrients (Edgar, 1992). Furthermore, physico-chemical properties such as pH, buffering capacity and state of saturation with respect to enamel mineral, affect the prevailing environmental conditions (Pearce, 1991), and subsequently may influence bacterial metabolism. Saliva also contains both specific and non-specific immunological factors, (as discussed in Section 1.5.3) which are potentially bactericidal (Dawes, 1984; Smith & Taubman, 1991).

1.5.5 Influence of dietary factors on the cariogenic potential of oral microorganisms.

Diet plays a central role in the development of dental

caries (Sreenby 1982, Theilade & Birkhed, 1986), as reviewed in Section 1.2.2. The following paragraphs survey the evidence that dietary components may influence the cariogenicity of bacteria. The cariogenic potential of foodstuffs themselves differ and are related to a number of factors (Gustafsson et al., 1954), principally the content of fermentable carbohydrates and of sucrose in particular (Burt & Ishmail, 1986).

Sucrose acts as a substrate for the production of both intracellular and extracellular polysaccharides, and has been shown to influence the microbial composition of dental plaque (Donoghue & Perrons, 1991). Whilst it has long been recognised that the numbers of oral lactobacilli are influenced by dietary carbohydrate intake, (Jay, 1947), evidence relating to the effect of dietary factors on the concentration of mutans streptococci is inconsistent. Svanberg (1980) showed that the proportion of S. mutans in plaque increased following rinsing with a mouthwash of low pH, and a similar effect was achieved in student volunteers consuming sucrose-rich diets, the effect being greatest in the early stages of plaque maturation (Staat et al., 1975). Similarly, Minah, Solomon and Chu (1985), reported that sucrose intake was positively related to the concentration of S. mutans in molar fissures. However, Macpherson and co-workers (1990) failed to show significant differences in percentage or absolute counts

of organisms, when sucrose was applied to plaque on an intra-oral appliance over a three week period. Furthermore, there is evidence that despite regular perturbations to the environment, the overall community of dental plaque remains relatively constant (Dent & Marsh, 1981).

Sugar substitutes, such as the sugar alcohols, have been shown to reduce the cariogenic potential of bacteria (Theilade & Birkhed, 1986). However, Söderling et al. (1991), have reported that whilst the numbers of mutans streptococci in plaque of habitual xylitol consumers were about ten percent of those in controls, salivary numbers of these organisms were relatively high in both groups. The effect of adding fluoride to the diet on caries formation has been extensively reported (Newbrun, 1989). Nonetheless, the result of adding other caries-protective agents, such as phosphate, to the diet have not been shown to affect the cariogenic potential of bacteria in humans (Theilade & Birkhed, 1986).

1.5.6 Influence of chemotherapeutic agents on the cariogenic potential of oral microorganisms.

The cariogenic potential of oral microorganisms may be influenced by antimicrobial agents. Whilst it has long been known that antibiotics will inhibit dental caries in experimental animals (Mc Clure & Hewitt, 1946), and penicillin, tetracycline, spiramycin and erythromycin

have all been shown to inhibit plaque formation as reviewed by Kidd & Joyston-Bechal (1987), the potential side-effects prohibit the widespread use of antibiotics to specifically control dental caries. It is interesting that Loesche et al. (1989), have suggested that medically administered antibiotics may be implicated in recently observed caries prevalence changes.

The inhibitory effect of chlorhexidene on dental plaque was first reported by Löe and Schiott (1970), and this agent has been used successfully to influence the cariogenic potential of microorganisms in xerostomic patients (Fure & Emilson, 1990). The use of chlorhexidene on a long-term basis to eliminate mutans streptococci in normal patients has, however, been questioned (Rölla, 1986).

The antimicrobial effects of fluoride have been extensively investigated and have been reviewed by (Hamilton & Bowden, 1988). However, the effect of this ion on the cariogenicity of oral microorganisms is often regarded as of little or no importance when compared with the direct interactions of fluoride with the hard tissues during caries lesion development and progression (Clarkson et al., 1988). Van Louvren (1990), reported that, despite the ability of oral bacteria to adapt to fluoride, there is no evidence that this results in a reduced cariogenic potential in normal subjects. A

number of other chemical agents such as sanguinarine, phenols, quaternary ammonium compounds, surfactants and metallic salts have been investigated as anti-plaque agents (Seymour & Heasman, 1992). Watson, Cummins and van der Ouderaa (1991), have reported that ionic zinc was strongly inhibitory to *in vitro* acid production from glucose by washed cell suspensions of *S. mutans*.

1.6 Bacterial specificity and dental caries.

From the foregoing discussions, it is apparent that bacterial involvement in the caries process is complex and influenced by many factors. The question of the role played by individual bacterial species is important in our understanding of the disease. Many investigators have shown that plaques removed from diseased sites have characteristic microbial profiles that differ in significant ways from plaques removed from non-diseased sites. These findings led Loesche (1979), to advance the Specific Plaque Hypothesis, which declares that specific microbial species play key-roles in the initiation and progression of dental caries. This contrasts with the alternative view or Non-Specific Plaque Hypothesis, which holds that the initiation of a carious lesion is a result, not of the action of one, or even a small number of specific bacterial species, but is due to the activity of all acidogenic organisms that contribute to the pool of acid in plaque (Edwardsson, 1986).

The demonstration of a cause and effect relationship between microorganisms and a disease process has, for over a century, been based on the fulfilment of the criteria or "postulates", first proposed by Koch. These fundamental tenets of medical microbiology are, however, difficult to fulfil when the proposed aetiological agent is part of the commensal flora, and is only one factor in a disease process which is of multifactorial origin.
In order to examine the role of a specific bacterial species in enamel caries, it is necessary to consider a number of factors, namely; association with the disease; the effect of eliminating or reducing the number of the organism; the immunological response of the host to the organism; the ability to cause disease in experimental animals; the possession by the organism of biochemical characteristics of importance in the pathogenesis of the disease, and communicability between individuals (Socransky, 1979; Emilson & Krasse, 1985).

As discussed previously, mature plaque is composed of a complex ecosystem, which consists of many bacterial genera and species which vary, from time-to-time, from site-to-site, and from individual to individual. Van Houte (1980), has suggested that direct proof of the causative role of any given plaque organism could only be obtained by eliminating it selectively from the mouth, or by preventing its initial oral establishment in conjunction with an appropriate clinical trial, both of

which would of course, be extremely difficult - if not impossible to achieve.

The question of determining bacterial specificity in caries is complicated further by the fact that dental caries is, in its initial stages, a reversible process involving both the demineralisation and remineralisation of tooth tissue (Silverstone 1977). Additionally, it is difficult to identify caries in its earliest stages and correlate changes in the bacterial community with changes in underlying tooth structure (Marsh et al., 1989).

Nonetheless, a great deal of information on the role of specific species in caries aetiology has been obtained in the past thirty years. Studies have taken the form of cross-sectional and longitudinal epidemiological investigations; in vivo studies in animal models (Fitzgerald & Keyes, 1960; Krasse & Carlsson, 1970; Drucker, Fitzgerald & Green, 1981); in humans, using intra-oral appliances (Brudevold et al., 1984; Tehrani et al., 1986; Macpherson et al., 1990); or devices for artificially creating caries in teeth due for extraction (Holmen et al., 1985; Boyar et al., 1989), and from in vitro artificial mouth type studies (Noorda et al., 1988).

1.7 The mutans streptococci and dental caries.

1.7.1 Introduction.

S. mutans is a non-motile, catalase negative, gram positive coccus (Clarke 1924), and its role in human dental decay has been reviewed extensively by Hamada and Slade (1980), and Loesche (1986). The species was first described by Clarke (1924), who isolated it from 36 of 50 carious lesions investigated. The streptococcus had very distinctive characteristics, and the name Streptococcus mutans was proposed. Almost 40 years passed before interest was again focused on the role of S. mutans in dental caries. Fitzgerald and Keyes (1960) demonstrated the aetiological role of certain streptococci in experimental caries in hamsters, which were later identified as S. mutans (Carlsson, 1967).

1.7.2 Taxonomy of the mutans streptococci.

Whilst, until recently, the term S. mutans was commonly used in the literature, it has become clear (and is officially accepted, Coykendall, 1983), that the bacteria formally known collectively as S. mutans, display considerable serological (Bratthall, 1970; 1972), biochemical (Shklair & Keene, 1974), and genetic heterogeneity (Coykendall, 1974; Ivic & Russell, 1988; Caufield & Walker, 1989), and are now recognised as S. cricetus; S. downei; S. ferus; S. macacae; S. mutans; S. rattus and S. sobrinus, the organisms being collectively known as the mutans streptococci (see Table 1.1).

	S. cricetus	*S. rattus*	*S. mutans*	*S. sobrinus*
Serotype	a	b	c,e & f	d & g
Mol % Guanine + Cytosine	43-44	41-43	36-38	44-46
Hydrolysis of Arginine	-	+	-	-
Fermentation of				
Raffinose	+	+	+	-
Melibiose	+	+	+	-
Inulin	+	+	+	-
H_2O_2 production	-	-	-	+
Aerobic growth	-	+	+	+
Bacitracin sensitivity	+	-	-	±

	S. ferus	*S. macacae*	*S. S.downei*
Serotype	c	c	h
Mol % Guanine + Cytosine	43-45	35-36	40-42
Hydrolysis of Arginine	-	-	-
Fermentation of:			
Raffinose	-	+	-
Melibiose	-	-	-
Inulin	+	-	ND
H_2O_2 production	-	-	+
Aerobic growth	+	+	+
Bacitracin sensitivity	+	+	-

+ = positive
- = negative
ND = Not determined.
Mol = moles

Table 1.1. Characteristics of the mutans streptococci. (Adapted from De Soet, 1990).

1.7.3 Isolation of mutans streptococci.

Mutans streptococci can be isolated readily from dental plaque and saliva by means of selective media. However the adequacy of various selective media in the isolation and identification of mutans serotypes, in particular S. mutans and S. sobrinus, has recently been the cause of debate.

Mitis Salivarius Bacitracin (M.S.B.) (Gold, Jordan & van Houte 1973), is the selective medium most commonly used to isolate mutans streptococci. This media has, however, been reported to inhibit the growth of S. sobrinus and S. cricetus (Tanzer et al., 1984; Wade, Aldred & Walker, 1986). Alternative selective media such as trypticase yeast cystine sucrose bacitracin (T.Y.C.S.B. - van Palenstein Helderman, Ijsseldijk & Huis in 't Veld, 1983), have been reported to be superior to M.S.B. for the isolation of S. sobrinus, as well as S. mutans. However, Holbrook and Beighton (1987), and Svanberg and Krasse (1990), favoured M.S.B. for the isolation of mutans streptococci.

More recently described methods of detecting mutans streptococci include a latex agglutination test, in which antibody-sensitised latex particles were used to detect mutans streptococci in dental plaque (Takei, 1991). De Soet et al. (1989) have reported a monoclonal antibody system for detecting S. sobrinus. However, no reports have yet appeared in which such an assay has been used to

identify fresh isolates in a large scale epidemiological study (Russell, 1991).

1.7.4 Epidemiology of mutans streptococci.

In 1968, Krasse and co-workers reported a significant correlation between the number of S. mutans isolated from saliva and caries activity (Krasse et al., 1968). In the interim, the relationship between the mutans streptococci and dental caries has been investigated extensively, resulting in many persons, both within and without the dental profession, considering S. mutans as synonymous with dental caries (Beighton, 1991). However, increasingly, epidemiological evidence has confirmed the complexity of the relationship between the mutans streptococci and dental caries.

Numerous cross-sectional studies, (Littleton, Kakehashi & Fitzgerald, 1970; Englander & Jordan, 1972; Hoerman et al., 1972; Weinberger & Wright, 1989; Twetman & Frostner, 1991), have reported significant correlations between S. mutans levels and caries experience and, in a number of longitudinal studies (Ikeda, Sandam & Bradley, 1973; Keene & Shklair, 1974; Klock & Krasse, 1978), significant increases in S. mutans levels, prior to the initiation of smooth surface caries were recorded. However, Hardie et al. (1977) reported that, after a two year period, of 15 approximal surfaces examined no single bacterial species was uniquely associated with the onset of the disease. In a study of the microflora associated with developing

lesions of nursing caries, Milnes and Bowden (1985), observed increases in levels of S. mutans associated with the development of white spot lesions and cavities. However caries-free sites showed similar increases in the absence of lesion formation.

The microbiology of early caries lesions in vivo on the approximal surfaces of premolars extracted for orthodontic reasons, below orthodontic bands and of enamel sections mounted on an intra-oral appliance, has been investigated by Marsh et al. (1989); Boyar et al. (1989), and Macpherson, MacFarlane and Stephen (1990). These authors observed demineralisation in the absence of mutans streptococci in 37%, 23% and 27% of cases respectively. Clarke (1924) failed to detect S. mutans in 36% of the teeth he originally investigated, a fact which has been overlooked in much of the literature. It is therefore apparent that teeth may harbour mutans streptococci without becoming carious and, conversely, teeth may become carious in the absence of detectable mutans streptococci.

Interest has recently focused on the relationship of specific species of mutans streptococci and caries experience. The prevalence of these species varies between human populations, but S. mutans (serotypes c, e and f) accounts for about 70% to 100% of isolates, S. sobrinus (serotypes d and g) being the second most

commonly isolated species. The remaining species were primarily isolated from animal plaque, although S. rattus and S. cricetus have occasionally been isolated from human samples (Brathall, 1972; Shklair & Keene, 1976; Hamada & Slade, 1980). Killian, Thylstrup and Fejerskov (1979) reported high levels of mutans streptococci in caries-free Tanzanian children, but Loesche (1986), in support of the Specific Plaque Hypothesis, reported that 83% of these isolates were serotype b (S. rattus), and proposed that this species may be less cariogenic than S. mutans or S. sobrinus. Carlsson et al. (1987) isolated mutans streptococci (of which 88% were S. mutans and 12% S. sobrinus) from 96% of a rural Sudanese population, 96% of whom were caries-free, and concluded that mutans streptococci can be widespread in human populations with an extremely low prevalence of dental caries.

De Soet (1990), claimed that underestimation of S. sobrinus may contribute to reduced associations of mutans streptococci and caries. Lindquist and Emilson (1991) investigated differences in the distribution and prevalence of S. mutans and S. sobrinus in 40 Swedish adults harbouring both species. No significant differences could be found in the relative proportions of these species between sound, decayed or filled surfaces. However speciation in this study was based on colonial morphology. The Third European Oral Microbiology Workshop concluded that speciation of S. mutans and S. sobrinus in assessing caries risk, was not considered to

be cost-effective in the United Kingdom or the United States of America, where the frequency of isolation of S. sobrinus is less than ten percent (Bagg & Kinane, 1990).

1.7.5 Virulence of mutans streptococci.

It is recognised widely that the mutans streptococci possess many of the phenotypic traits considered important in determining cariogenic potential (Hamada & Slade, 1980; Loesche, 1986). Primarily, these consist of factors that promote the adherence of bacteria to dental enamel and plaque, and factors involved in the production of acids (Hamada, Koga & Ooshima, 1984).

Mutans streptococci are capable of producing extracellular polysaccharides from sucrose (Gibbons, 1986). Glucans of two distinctive types can be produced, namely an α-(1-3)-linked water insoluble and an α-(1-6)-linked water soluble polymer, catalysed by glucosyl transferases GTF-I and GTF-S respectively (Hamada & Slade, 1980). Both of these polymers have been implicated in the adherence of strains to tooth surfaces and in contributing to the structure of dental plaque. These materials may influence the cariogenic potential of dental plaque by altering its diffusion characteristics (Zero, Van Houte & Russo, 1986).

Mutans streptococci are also capable of storing polysaccharides intracellularly (Gibbons & Socransky

1962). Such stores may increase the cariogenic potential of bacteria by contributing to acid production in the absence of exogenous sugar (De Stoppelar, Van Houte & Backer Dirks, 1969; Tanzer et al., 1976).

The aciduric and acidogenic properties of mutans streptococci are considered to be major determinants of the cariogenic potential of these species (Loesche 1986), and have been investigated extensively. The ability of bacteria in plaque to produce acid varies. When first exposed to carbohydrates all acidogenic bacteria produce acids. However, as the pH decreases, an increasing number of the bacteria loose this ability and, at the so-called critical pH value, when the tooth surface experiences nett mineral loss, only the most acidogenic bacteria produce acids. Clearly, the latter may be of major importance in the pathogenesis of caries (Edwardsson, 1986). There is much evidence from both in vitro (Harper & Loesche, 1983; Kaufman et al., 1984; Carlsson, 1986; De Soet, Toors & De Graff, 1989) and in vivo studies in animal models (Drucker, Fitzgerald & Green, 1981; Willcox et al., 1985) that, with the possible exception of Lactobacillus species, the ability of the mutans streptococci to produce acids at low pH levels exceeds that of most other oral microorganisms.

Recently, debate has focused on differences in the cariogenic potential of the individual species of mutans

streptococci. Whilst S. mutans and S. sobrinus have been reported as more cariogenic than other species in the group, studies vary in their conclusions as to whither S. mutans or S. sobrinus represents the ultimate cariogenic challenge (Kholer & Krasse, 1990; De Soet et al., 1991). Less evidence has, however, been reported on the variation in the acidogenic potential of different strains of S. mutans (Harper & Loesche, 1983; Macpherson, 1988; Macpherson et al., 1992).

1.8 Lactobacillus species and dental caries.

Lactobacilli are gram positive, non-spore forming rods as reviewed by Newbrun (1989), with L. casei and L. fermentum being the most commonly isolated oral species. Of the virulence factors discussed in Section 1.5.1, it is the aciduric and acidogenic properties of this genus that has lead specifically to their implication in the carious process (Ellen, 1976).

Edwardsson (1986), cited studies by Goadby (1903) and Kligher (1919) which described, respectively, the isolation of Bacillus necrodentalis from carious lesions, and the demonstration of higher proportions of Lactobacilli-like microorganisms in plaque from carious surfaces than from plaque on caries-free surfaces. Bunting, Nickerson and Hard (1926), claimed that Bacillus acidophilus was the specific aetiological factor responsible for the initiation of caries, and Rodriguez (1931) demonstrated a significant correlation between

Lactobacillus acidophillus (Bacillus acidophilus) and dental caries.

Until the 1960's, the view that Lactobacillus species were the principal organisms in caries aetiology dominated the literature. However, as interest focused on the cariogenic potential of Streptococcus species, the contribution of lactobacilli to the disease was re-evaluated. The numbers of lactobacilli in plaque and saliva have been shown to be influenced by ecological determinants (Ellen, 1976). For example lactobacilli generally have difficulty in attaching themselves to smooth surfaces, and it may be that an acid environment, together with the presence of extracellular polymers or a mechanical retention site, may be a prerequisite for colonisation of Lactobacillus species, (Socransky & Manganiello, 1971; van Houte, Gibbons & Pulkkinen, 1972). Ikeda et al. (1973) stated that lactobacilli were apparently not essential for the initiation of carious lesions, and it is probable that plaque conditions which are conductive to caries, also favour colonisation by these species (Crossner, Claesson & Johansson, 1989). Furthermore their presence in carious lesions appear to be secondary to initial enamel breakdown by other plaque bacteria.

Recently, the question of the cariogenic potential of individual species of Lactobacilli has been raised.

Beighton (1991), stated that the role of individual species has not been considered even though the diversity of the Lactobacillus species is great, and recent taxonomic studies have underlined our ignorance of oral lactobacillus populations. However, Crossner et al. (1989), reported no obvious differences in the percentage of various Lactobacillus species between interdental spaces with, and without, subsequent caries progression.

1.9 Veillonella species and dental caries.

Veillonella species are anaerobic gram negative cocci which have been demonstrated in the deeper layers of dental plaque (Ritz, 1967; Ritz, 1969). The precise contribution of this genus to dental caries is unclear. Veillonella have been shown to utilise lactic acid generated by plaque during glycolysis, converting it to the weaker propionic and acetic acids (Distler & Kroncke, 1981).

It has been proposed that in dental plaque, Veillonella species may live in a commensal relationship with lactic acid-producing organisms, thereby reducing their cariogenic potential (Mikx & van der Hoeven, 1975). When V. alcalescens was combined with S. mutans in gnotobiotic rats, the caries scores were significantly reduced as compared with S. mutans alone (Mikx et al., 1972). However, more recently, no inhibition of demineralisation was observed in vitro when V. alcalescens was cultured with S. mutans in an "artificial mouth" (Noorda et al.,

1988), and the original concept of the role of Veillonella has been questioned.

Clinically, higher proportions of Veillonella have been found at progressing incipient lesions (Boyar & Bowden, 1985), and at sites with nursing bottle caries (Milnes & Bowden, 1985). Marsh et al. (1989), in a study of early caries on approximal surfaces of premolars in schoolchildren, also found the highest proportions of Veillonella at sites with mutans streptococci and early caries. However, the lowest proportions of Veillonella were found at those sites that had developed early caries in the absence of S. mutans. These workers proposed that Veillonella respond to, and their proportions in plaque reflect the fluctuating concentrations of, lactate at a site, but that when Veillonella are either absent or present only in low numbers, they allow the cariogenic potential of plaque bacteria other than mutans streptococci to be expressed.

1.10 Yeasts and dental caries.

In comparison with other groups of microorganisms, the cariogenic potential of yeasts has received little attention. A relationship between yeasts both in saliva and plaque, with dental caries, has been observed (Krasse, 1954; Brown et al., 1978; Pienihäkkinen et al, 1987; Russell et al., 1990). These organisms are aciduric but produce acid relatively slowly. In healthy

dentate persons, the primary oral reservoir is the tongue, and their numbers in dental plaque are low. It is therefore likely that, rather than contributing directly to the initiation of dental caries, the association of yeasts with the disease reflects their aciduric properties (Edwardsson, 1986).

1.11 Other Streptococci and dental caries.

The predominant bacteria in human dental plaque are members of the genus Streptococcus. Whilst many investigations have concentrated on the role of the mutans streptococci in dental caries, less attention has been paid to the cariogenic potential of other species of Streptococci. Recent studies (Boyar et al ., 1989; Marsh et al., 1989; Macpherson et al., 1990) have observed early carious lesions developing in the absence of detectable levels of mutans streptococci. This raises the question of the cariogenic potential of other streptococcal species. Early studies on germ-free animals maintained on a highly cariogenic diet, demonstrated the relative cariogenicity of various Streptococci, (Fitzgerald, Jordan & Stanley, 1960; Fitzgerald, 1968). Fitzgerald (1968) claimed Streptococci are generally more cariogenic than other genera, but not all Streptococci are equally cariogenic (Drucker, Fitzgerald & Green, 1981; Drucker & Green, 1981). These workers have shown that S. milleri, S. oralis and S. bovis can produce severe to moderate levels

of dental caries in rodents. However the lesions are usually confined to the pits and fissures of the teeth.

1.12 Dental plaque and dental caries.

Bacteria colonise the teeth in the form of dental plaque, and Löe (1969), reported that the presence of plaque was required for caries development in humans. It is evident that caries occurs most frequently on those tooth surfaces where plaque accumulates most readily, and in the highest amounts. However most clinicians are familiar with the findings of de Paola et al. (1968), and Andlaw (1978), who described individuals who remain caries-free in spite of unfavourable oral hygiene.

The cariogenic potential of plaque is influenced by the varying abilities of its constituent microorganisms to produce acid or basic end products, to tolerate an acid milieu, to degrade acid end-products, or to influence the composition of the plaque matrix which may play a role in the retention of bacterial acids (van Houte 1980). In a now famous experiment, Stephan (1944), investigated the in vivo pH response of dental plaque after rinsing with a carbohydrate solution, and demonstrated that "resting" pH values, as well as the lowest pH obtained and the rate of acid production, were related to caries activity. Subsequently, a number of workers have attempted to correlate the cariogenic potential of dental plaque with

clinical caries activity. The results of these studies are, however, inconsistent.

Vratstanos and Mandel (1982) studied the comparative plaque acidogenesis of caries-resistant and caries-susceptible adults. They reported that the amount, and rate of production of lactic acid were lower in the caries-resistant group, and that their levels of acetic acid were significantly higher. They proposed that the cariogenic potential of plaque was influenced by the presence of high pK_a acids (such as acetic, propionic and butyric) which can provide a buffering system (acetate-acetic acid) capable of countering the pH decrement generated by the low pK_a acids (lactic, formic and pyruvic). Margolis, Duckworth and Moreno (1988a) were unable to demonstrate significant differences in the total concentration of high pK_a acids in resting plaque fluid of caries-free and caries-susceptible groups. However, these workers reported higher pH and NH_4^- concentrations in the caries free group. In a companion study, Margolis, Duckworth & Moreno (1988b) were unable to detect significant differences in the composition, saturation status or inherent buffering capacities, of pooled, starved plaque fluid from caries-free and caries-susceptible individuals. Neither of these studies, however, precluded the possibility that, following exposure to fermentable carbohydrates, differences in the cariogenic potential of dental plaque would become

apparent, correlate with caries-susceptibility or reflect the presence of particular bacterial species.

1.13 The epidemiology of dental caries.

1.13.1 Changes in caries prevalence.

Archaeological evidence has demonstrated that dental caries has afflicted man since prehistory and, whilst much less common in earlier periods than it is today, prevalence of the disease varied throughout the ages (Lunt, 1974). In Britain, during the eighteenth and first half of the nineteenth centuries, dramatic increases in caries prevalence were observed, carious lesions becoming common on occlusal and interproximal surfaces, and affecting younger age groups, in contrast to the cervical caries affecting adult dentitions in medieval and earlier populations. Moore (1983), ascribes these changes to the well documented alterations in contemporary diet.

In the first half of the present century, the prevalence of dental caries in industrialised societies increased to pandemic proportions (Downer, 1984). This rise was associated closely with the increased availability of refined carbohydrates, and of sucrose in particular (Marthaler, 1990). However, data from many countries (assimilated by the World Health Organisation and reviewed by Renson et al., 1985), show that over the past two decades, the prevalence of the disease in children

resident in developed countries, namely Scandinavia, Western Europe, North America, and Australasia, has declined. A number of reasons have been postulated to explain this decline, but prominent amongst them is the increasing availability and use of fluoridated dentifrices, (Glass, 1982; Holm, 1990). However, recent evidence amongst five-year-olds (Downer, 1989; Rugg-Gunn et al., 1989), suggests that the decline may have plateaued, and a number of authors have warned against complacency in regarding dental caries as a declining disease (Johnson, 1991; Kidd, 1991).

Thus, whilst dental caries has traditionally been regarded as a disease of the young, it is possible to speculate that the progress of the disease to the cavitation level may be delayed to adulthood. Furthermore, as enamel caries declines, it is likely that the increasing proportion of the population retaining teeth into old age, will result in an increased prevalence of root caries (Wagg, 1984; Winter, 1990).

In contrast to the decline in caries prevalence in developed countries, in many developing nations which have previously experienced a low disease activity, the incidence of dental caries is rising. As Johnson (1991) comments, this represents an alarming situation, as some 80% of the Worlds' population live in developing countries. It is possible, therefore, that at present

the early stages of a global dental caries epidemic is occurring.

1.13.2 Concept of High Risk Groups.

The changes in caries prevalence in Western countries (discussed in Section 1.13.1), relate to populations as a whole. However, not all individuals have benefitted equally from the decline in disease levels. In the period 1950-1970, caries experience was regarded as being distributed normally throughout a given population. However, as caries prevalence has declined, the distribution of the disease has become skewed (Spencer, 1986; Whelton, O'Mullane & Moran, 1986). Mandel (1989) has reported that epidemiologically, caries has become a dichotomous disease in children of most developed countries where, whilst up to 50% of the children may be caries-free, 20% account for 75% of lesions. Pitts (1991) reported that of 6500 amalgam restorations received by 1500 Scottish children between 1983 and 1988, 35% of the children received 74% of amalgams provided. It is becoming apparent therefore that there exist "high risk" cohorts within child populations, and increasing efforts are being directed at identifying those who fit into this category, (Winter, 1990; Johnson, 1991; Disney et al., 1992).

1.14 Caries-activity tests.

A basic goal in medicine and dentistry, is to prevent the

initiation of disease and its further development (Koch, 1988). As long as the caries prevalence in a population is generally high, most of the effort has to be directed to the application of preventive measures to the whole population. However, as the proportion of the population experiencing the disease decreases, the cost of caries-prevention activities could be greatly reduced, and their efficiency increased sharply, if the clinician or public health administrator were able to identify, in advance, those subjects or groups at greatest risk of developing the disease (Hunter, 1988).

The search for methods to predict caries-risk began last century (Hunter et al., 1988) and, in the intervening period, a multiplicity of caries-activity tests has been described in the literature. As noted by Newbrun (1989), this suggests two things: firstly that there is a very definite need for a good caries-activity test and, secondly, that none of the currently available methods are entirely satisfactory.

As discussed in Section 1.2, the aetiology of dental caries is multifactorial in origin, and the challenge of determining the relative risk posed to a caries-free individual within a population, or to a sound enamel surface on a given tooth in a particular mouth, is obvious.

A caries-activity test must either measure some factor related intimately to the disease process, or measure one that reflects accurately the disease process (Bowen, 1969). Data in Table 1.2 show some of the variables that have been investigated as determinants of caries-risk. In the search for methods resulting in good prediction, these variables have been studied singly and increasingly, in combination.

1.15 Potential benefits and uses of caries-activity tests.

The value of achieving an accurate caries-activity test in the light of contemporary changes in caries prevalence, have already been alluded to in Sections 1.13.2 and 1.14. However, potential benefits of such a test were outlined by Socransky (1968) as follows:

To the clinician,

1 Determination of the need for caries control measures.
2 Indicator of patient cooperation.
3 Aid in timing of recall appointments.
4 Guide to insertion of expensive restorations.
5 Helpful in the determination of prognosis.
6 Precautionary signal to the orthodontist.

-and-

To the research worker,

1 Aid the selection of patients for caries studies.
2 Screening of potential therapeutic agents.

Caries prevalence
Dental care attitudes
Dietary habits
Diseases
Microbiological data
Oral hygiene
Salivary factors
Structure and chemistry of the tooth
Socio-economic factors

Table 1.2 Variables that have been used as determinants of caries risk. (After Koch, 1988).

3 Indicator of periods of exacerbation and remission.

1.16 Ideal requirements of a caries-activity test.

Snyder (1951) proposed criteria for determining the value of a method of caries prediction. He stated that if a test was to be of value to a practising dentist, then it should fulfil the following requirements:

1 Have maximal correlation with clinical status.
2 Be accurate in respect to duplication of results.
3 Be rapid in performance.
4 Be simple.
5 Require minimum equipment.
6 Require minimum skill.
7 Have low cost per test.

To the foregoing requirements, Snyder later added that a correlation coefficient of 0.9 between the test and caries increments would demonstrate the usefulness of a test. Unfortunately, correlation coefficients have been misused in many investigations of caries activity tests. Poulsen and Holm (1980) stated that, whilst a correlation coefficient can be used to determine whether a statistically significant relationship exists between the predictor and the caries score, it does not indicate whether the relationship is so close that it can be used for selecting children with a high risk of developing caries. They suggest that a better estimation of the

efficiency of a caries-activity test were sensitivity and specificity, where sensitivity is defined as the percentage of truly diseased subjects correctly identified and specificity is the percentage of truly non-diseased subjects correctly identified (see Table 1.3). Obviously the ideal caries-activity test would have values of sensitivity and specificity of 100%. However, in practical terms, the aim is to have the maximum number of truly diseased subjects identified with the minimum number of truly non-diseased subjects included (Hunter 1988).

In recent studies, increasingly complex statistical methods such as multiple regression procedures, discriminant analysis, and Classification And Regression Tree analysis (Stewart & Stamm, 1991), are being applied to combinations of clinical, microbiological and interview data, to predict high or low risk individuals or groups (Worthington, 1991).

The limitations of current caries-activity tests when compared with the ideals just outlined, are discussed further in Section 1.18.

1.17 Past and present caries activity tests.

While a detailed reappraisal of the history of caries-activity tests is outwith the remit of this thesis, Table 1.2 outlines examples of the many variables that

Screening Criterion	Validating Criterion		
	Positive	Negative	Total
Positive	a (true positive)	b (false positive)	a+b
Negative	c (false negative)	d (true negative)	c+d
Total	a+c	b+d	n

SENSITIVITY = [(a/a+c) x 100] i.e. the probability that a screening procedure will give a positive finding which is in agreement with the validating criterion.

SPECIFICITY = [(d/b+d) x 100] i.e. the probability that a screening procedure will give a negative finding which is in agreement with the validating criterion.

DIAGNOSTIC POWER = [(a/a+b) x 100] also termed the positive predictive value i.e. the proportion of true positive among recorded positive cases.

Table 1.3 The definition of Sensitivity, Specificity and Diagnostic Power as used in the validation of caries activity tests. (After Krasse, 1988).

have been explored in the quest for a satisfactory caries-activity predictor. However, the following discussion will be limited to salivary and microbiologically based tests.

1.17.1 Salivary related caries-activity tests.

A number of tests aimed at relating the acidogenic potential of saliva and carbohydrate mixtures to caries-activity have been described (Fosdick, Hansen & Epple, 1937; Wach et al., 1943; Dewar, 1949; Rickles, 1953). The Fosdick test determines acid production by measuring calcium release from powdered enamel after a four hour incubation of a saliva, glucose and enamel mixture. The Dewar test was, in essence similar, but acidogenic potential was assayed by the pH attained after four hours' incubation. In a review of the then available caries-activity tests, Socransky (1968) deemed these early assays unsatisfactory in assessing caries risk.

The Snyder test (Snyder 1940), is based on the growth of microorganisms from stimulated saliva samples in an acidic agar containing medium. Here, cariogenic potential was assessed by the length of time necessary for the bacterial growth to change the colour of the pH indicator by producing a further decrease in the pH of the medium. Ellen (1976) reported that the Snyder test may be classified as a simplified Lactobacillus count. Whilst it is simple and inexpensive to conduct, and some

studies have shown a significant correlation between test results and caries-activity on a group basis (Snyder 1951), the test does not predict the extent of future caries development with any reliability for one individual (Newbrun 1989).

1.17.2 Salivary flow-rate and caries-activity.

The influence of the lack of saliva on dental caries in xerostomic patients as, for example, in Sjögrens' Syndrome, and following irradiation of the salivary glands, has been well documented (Dreizen & Brown, 1976). However, when one moves beyond these extreme situations to studies attempting to correlate the volume of saliva secreted per unit time with caries-activity, no consistently significant relationship can be observed (Klock & Krasse, 1979; Larmas 1985; Söderholm & Birkhed, 1988; Klock et al., 1989).

1.17.3 Salivary buffering capacity and caries-activity.

The buffering capacity of saliva has been shown to vary with caries-activity (Stople, 1970). Mandel (1978) claimed that salivary buffering capacity was the most likely of the inorganic constituents or properties of saliva to be of use as a caries-activity test. However this early promise has not been fulfilled. A recent study (Pienihäkkinen et al., 1987), using a commercially available test, Dentobuff (Orion Diagnostica, Finland), to measure salivary buffering capacity, showed at best

weak correlations with caries-activity, these findings being supported by Alaluusua et al., 1990. Newbrun (1989) reported that the inverse relationship between the buffering capacity of saliva and caries-activity was insufficient to permit its use as a clinically useful test.

Pearce (1991) discussed the reasons why salivary constituents and physical properties were poor predictors of future caries-activity. He concluded that despite fifty years of research, no inorganic or physical factor in saliva has been shown to correlate strongly with caries-activity. Dawes (1984) postulated that the fluid of importance during the dissolution episodes of dental caries is not saliva, but the fluid of dental plaque, and the influence of the overlying plaque on the composition of the plaque fluid. It is therefore becoming clear that salivary related factors are an insufficiently precise tool to evaluate caries risk, except perhaps in pathological states.

1.17.4 Microbiologically based caries-activity tests.

The earliest microbiological tests were designed either to enumerate acidogenic bacteria in saliva, or to measure the acid produced by samples of whole saliva incubated with various carbohydrates (Ellen, 1976). As in many areas of microbiological research, advances in microbiological caries-activity tests were closely

related to advances in the culture and characterisation of bacteria.

1.17.5 Lactobacillus species and caries-activity tests. The enumeration of salivary lactobacilli became the first accepted microbiological caries-activity test (Rodriguez, 1931) and, in the intervening period, the role of this species in determining caries risk was evaluated in numerous studies. Significant correlations between lactobacilli and caries experience have been reported by Becks, Jensen and Millarr, (1944); Crossner, (1981); and Alaluusua et al., (1990). A commercially available system for estimating salivary lactobacilli load was reported by Larmas (1975), in which undiluted saliva was run over a dip slide coated with a modified Rogosa SL agar, and the density of the resultant growth of colonies compared with a standard illustration, whereby an equivalent colony count was obtained. Despite these reports, there are many conflicting accounts of the usefulness of counting lactobacilli in forecasting caries-activity when applied to individual patients. This has been explained by the influence of ecological determinants on the numbers of Lactobacilli in saliva (Ellen, 1976; Honkala et al., 1984), as discussed in Section 1.8.

1.17.6 Mutans streptococci and caries-activity tests. Characteristics thought to contribute to the

cariogenicity of the mutans streptococci, and their role in caries aetiology, were discussed in Sections 1.5 and 1.7. The potential of these species to act as markers of disease activity has been investigated extensively.
A variety of techniques have been used to collect samples of saliva or dental plaque prior to enumeration of mutans streptococci. Commonly, stimulated saliva samples are used (Klock & Krasse, 1977; Söderholm & Birkhed, 1988; Russell et al., 1991), but other techniques include the use of sterile spatulae (Kholer & Bratthall 1979), or loops (Beighton, 1986). A dip slide method (Dentocult SM, Orion Diagnostica, Finland) has also been described (Jordan et al., 1987).

Numerous studies in the past 20 years have demonstrated significant correlations between numbers of mutans streptococci and subsequent caries experience. However, initial hopes for counts of mutans streptococci as an ideal caries-activity test remain unfulfilled. The correlations observed between numbers of mutans streptococci and caries risk, whilst permitting a degree of determination of caries risk on a group basis, and in particular, in identifying groups with low caries increment, are insufficiently precise to enable accurate identification of the high risk individual (Disney et al., 1992) . Manji et al., (1991) have recently declared that, despite the evidence for the potential aetiological role of mutans streptococci in dental caries, these

microorganisms are of no value as determinants (or predictors) of the disease.

1.18 Limitations of current caries-activity tests.

From the foregoing, it is apparent that there does not exist a clinically useful salivary or microbiological caries-activity test, which will accurately and reproducability permit identification of the high risk individual prior to initiation of the disease.

Whilst the various tests go some way to complying with Snyder's proposed criteria (Snyder, 1951), the correlation coefficients between the parameters investigated and caries increment remain consistently low, values of 0.2 or 0.3 being typical. In recognition of the multifactorial nature of the disease, and of the over-reliance of caries-activity tests on one aetiological parameter, recent studies have investigated the combination of a number of tests in an attempt to improve predictive potential. Bader et al. (1988) investigated nine predictors of caries increment, and concluded that such an approach warranted further investigation. These findings endorsed the conclusions of Vanderas (1986). However, Wilson and Ashley (1989), who investigated a combination of salivary buffering capacity, bacterial counts, sugar intake and caries experience as predictors of 2 year and 3 year caries increment, concluded that whilst salivary diagnostic tests had potential, they required further development

before they could be used with confidence in clinical practice. In one of the most comprehensive studies of currently available microbiological and salivary caries-activity tests, Russell et al. (1990), concluded that the highest correlation between any single test and caries prevalence was 0.36 (i.e. well below Snyder's 0.9). Whilst the correlations obtained between the parameters investigated and caries prevalence were generally consistent and significant due to the number of subjects studied, they were low and insufficient for individual caries diagnosis. When variables were studied in combination, using stepwise regression analysis, it was possible to improve the association of the results and caries prevalence. However, in attempting to identify the DMFS or DS experience of individual children into high, medium or low groups, they reported, at best, only an improvement of 16% in overall identification rate was produced by using microbiological and salivary data. They concluded that even in combination, the caries-activity tests investigated were insufficiently accurate to identify an individual's caries-activity.

The potential of mutans streptococci to act as markers of disease activity have been investigated mainly in terms of the number of organisms present. Whilst, as discussed in Section 1.7.5, some consideration has been given to possible differences in the cariogenic potential of different species of mutans streptococci, little

attention has been paid to potential differences in the cariogenicity of individual strains of S. mutans. Since 90% of mutans isolated from children in the United Kingdom are S. mutans (Beighton, Rippon & Thomas, 1987), it was thought possible that the low correlations observed between numbers of S. mutans in saliva and caries-activity, could be improved by taking into account differences in the virulence of the strains. Thus, it may be possible to show that strains of S. mutans isolated from children with high counts of the organism and low caries scores, were less virulent than strains isolated from children with a lower bacterial count, but high disease experience.

This theory was supported to some extent by the work of Macpherson (1988) and Macpherson et al. (1992), who, in a pilot study, claimed that the in vitro cariogenic potential of six strains of S. mutans correlated significantly with the clinical caries experience (expressed as DMFS scores) of the six adults from whom the strains were originally isolated.

It was therefore proposed that further investigation of the cariogenic potential of oral microorganisms, and of S. mutans in particular, may further our understanding of current caries-activity tests, and formed the basis for the studies described in this thesis, as described below.

1.19 Aims of the current study.

The aims of the initial studies were to investigate further the model described by Macpherson (1988), as used to determine *in vitro* cariogenic potential (Chapters 2 and 3).

Subsequently the study described in Chapter 4 (using the model developed in Chapter 3), aimed to investigate the cariogenic potential of six species of streptococci.

The experiments conducted in Chapter 5 were designed to investigate the *in vitro* cariogenic potential of dental plaque collected from adolescents participating in a clinical dentifrice trial, and to relate the results obtained to clinical disease experience.

Studies described in Chapters 6-8 were designed to investigate further the variation in cariogenic potential of strains of *S. mutans* isolated from adolescents and to determine the possibility of using this technique to improve both the sensitivity and specificity of caries-activity tests, as discussed in Section 1.18.

CHAPTER 2

MICROBIOLOGICAL METHODS AND ANALYTICAL TECHNIQUES.

2.1 Introduction.

This Chapter initially describes the source, isolation, identification, storage and retrieval of clinical isolates used in the study. It then outlines the principles, practice and evaluation of the main analytical techniques employed in the assessment of cariogenic potential. Whilst the source of materials, manufacturers of instruments etc. are indicated in the text, details of the constituents and preparation of bacteriological media can be found in the Appendices. Throughout the study, the materials and methods used are as initially referred, unless otherwise indicated.

2.1.1 Lanarkshire Clinical Trial.

The majority of clinical specimens used in this study were isolated from 12 year olds participating in a three year double blind clinical dentifrice trial. In conjunction with Unilever Dental Research, Portsunlight, England, this project was conducted in Lanarkshire, Scotland, between August 1988 and March 1992. With the permission of the local ethics committee, 8353 children attending 32 secondary schools in the Lanarkshire Health Board Area were invited to participate in the study. Written parental consent was obtained for 6212 children and, after a clinical examination, 4294 were selected for inclusion in the trial on the grounds of past caries

experience and dental maturity. These examinations were conducted in accordance with FDI guidelines (FDI 1982) by two trained and calibrated clinicians. They were performed in convenient classrooms using transportable equipment, and participants were examined supine on a school table. Illumination was provided by a 55W Daray Light, placed approximately 20 cm from the subject's mouth. Examinations were undertaken without drying the teeth, using a No.5 Plane mirror and CPITN-C periodontal probe (Ash Instruments, Dentsply, Gloucester, England.). Tooth presence, plaque, gingivitis and calculus scores, in addition to caries and restoration status, were dictated to a trained scribe who recorded the information on a computer compatible form. The children were examined clinically on four occasions at annual intervals. In addition, during the baseline and final examinations, bitewing radiographs were taken, provided the child indicated that they had not been subjected to radiography in the previous six months. After the initial examination participants were stratified on the basis of sex, examining clinician, and dental parameters, (i.e. caries experience and dental maturity), before random allocation to one of six test product groups.
This trial therefore presented the opportunity to obtain bacteria from children with a range of known caries experience, and for whom incremental data would be available. Clinical samples were obtained during the

baseline and second examinations as described in Sections 2.2.1 and 5.1.2.

2.2. Microbiological Procedures.

2.2.1 Collection of saliva.

Children with a range of past caries experience were selected and a paraffin stimulated saliva sample collected. Samples were donated between 3 pm and 3.30 pm, and any child who had eaten in the previous ninety minutes, or had received antibiotic therapy within the past six weeks, was excluded. Subjects were asked to chew a pellet of paraffin wax (Orion Diagnostica, Helsinki, Finland.), until it became pliable. They then swallowed any saliva which had collected in their mouth and were instructed to chew for a further five minutes. All saliva produced during this period was voided to a pre-labelled sterile universal container. At the end of the collection period the container was capped securely and the wax disposed of aseptically (Figure 2.1).

The samples were then stored on ice, transported to the microbiology laboratory, Glasgow Dental Hospital, and processed within three hours of collection.

2.2.2. Isolation and enumeration of mutans streptococci and <u>Lactobacillus</u> species.

The total volume of saliva collected was measured using a Pipet-aid (Becton Dickinson Labware, Oxford, England.),

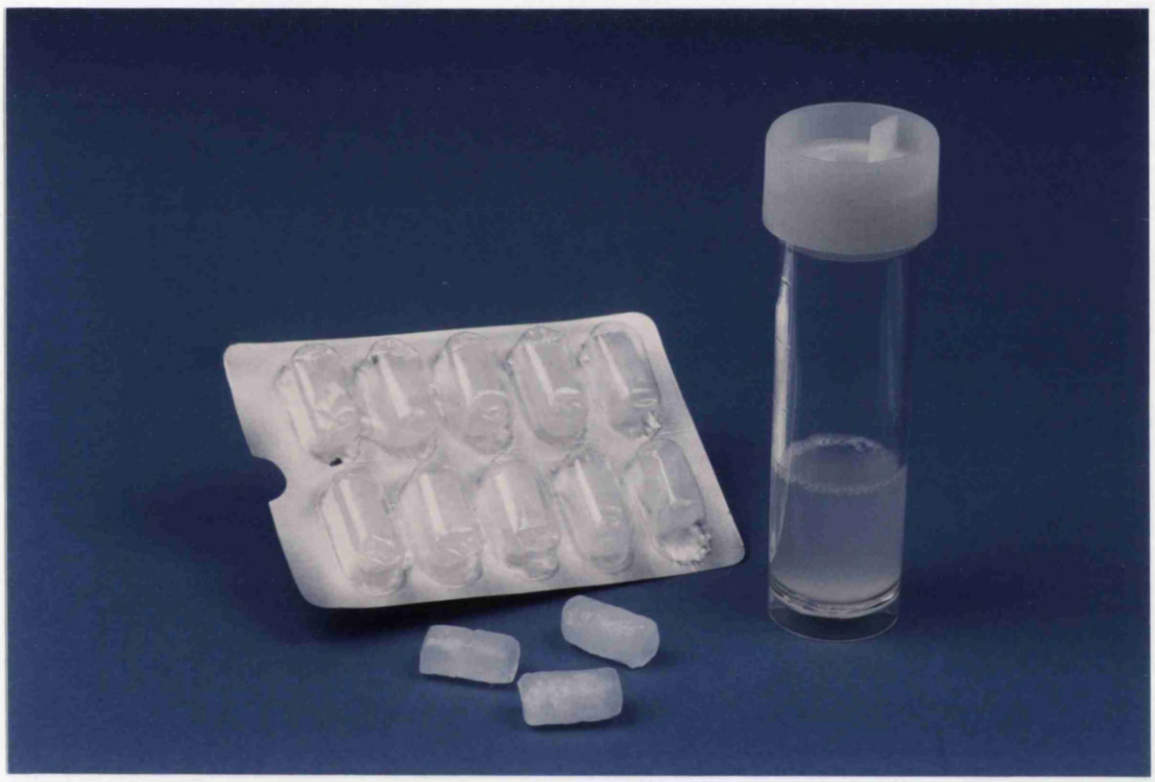


Figure 2.1. Stimulated saliva sample and paraffin wax.

thus the stimulated salivary flow rate could be calculated. A one millilitre sample of saliva was transferred aseptically to nine millilitres of sterile phosphate buffered saline (PBS), pH 7.2, (Flow Laboratories, Irvine, Scotland). Serial dilutions were performed by sequential transfer of one millilitre aliquots to a final concentration of 10^{-4}. By means of a spiral plater (Spiral Systems, Cincinnati, USA), 50 µl of the salivary samples were inoculated on to the appropriate selective culture media (Figure 2.2). Mutans streptococci species were isolated on Mitis Salivarius Bacitracin (MSB), (Gold, et al, 1973), and Lactobacillus species were isolated on Rogosa agar (Rogosa, Mitchell & Wiseman, 1957). Following inoculation, all cultures were incubated for 48 hours in an atmosphere of 5% CO_2 at 37°C (Qualitemp 80 MI Incubator, Laboratory Thermal Equipment, Oldham, England.), when a plate of appropriate dilution was selected and a colony count performed as detailed below.

The spiral plater dispersed the 50 µl on the surface of the agar plate in the form of an archimedean spiral, and the manufacturer supplied grid was superimposed to delineate sectors of different size in which a known volume of inoculum had been dispersed. A culture plate which contained 30 to 300 colonies was selected, and by simple arithmetic the number of colony-forming units per millilitre of saliva was calculated.

Figure 2.2. The inoculation of Mitis Salivarius Bacitracin by a spiral plater.

2.2.3 Identification of bacterial isolates.

After enumeration of mutans streptococci and Lactobacillus, five representative colonies of each were subcultured on to Columbia blood agar plates (supplemented with 7.5% defibrinated horse blood and 1% vitamin K and haemin solution; Gibco, Paisley, Scotland), and incubated at 37°C in an atmosphere of 5% CO_2 for 24 - 48 hours. The purity of the subcultured strains were confirmed by careful visual examination of the colonies. When more than one type of colonial morphology was observed, representatives of each were subcultured and subsequently identified on the basis of morphological and biochemical features. Gram stained smears (Gilles & Dodds 1976) were examined and mutans streptococci were characterised by API 20 Strep. identification galleries (API Montalieu, France). A combination of Minitek (Becton Dickinson, Oxford, England) and API 20A System were used to identify Lactobacillus species (Figure 2.3). Reclassification of the viridans streptococci has imposed limitations on the use of the API 20 system. Therefore additional information on the biochemical fermentation patterns of strains investigated in this study was gained using a modification of the method of Shklair and Keene (1974). The basal medium for fermentation was thioglycollate, to which was added bromocresol purple and one of the following carbohydrates; glucose, mannitol, sorbitol, raffinose, or melibiose. The preparation of this medium is described in Appendix A. A suspension of

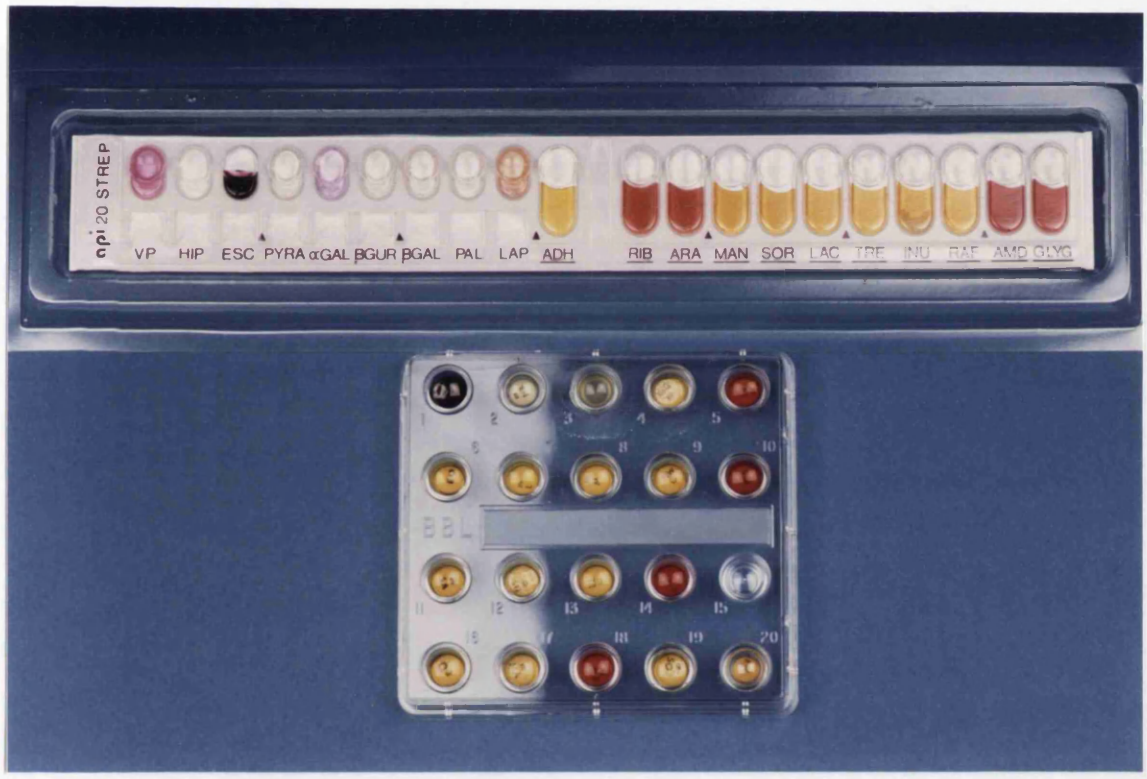


Figure 2.3. API 20 Strep and Minitek identification galleries.

the organism under investigation was prepared in phosphate buffered saline by the transfer of the entire growth from a heavily inoculated blood agar plate. The carbohydrate supplemented media was dispersed in microtitre plates, (0.3ml per well), and one drop of the bacterial suspension was added to each well using a sterile Pasteur pipette. The plate was covered with foil, and following incubation for 24 hours at 37°C in an atmosphere of air and 5% CO_2 the fermentation reactions were assessed by the colour change in the bromocresol purple indicator (Figure 2.4).

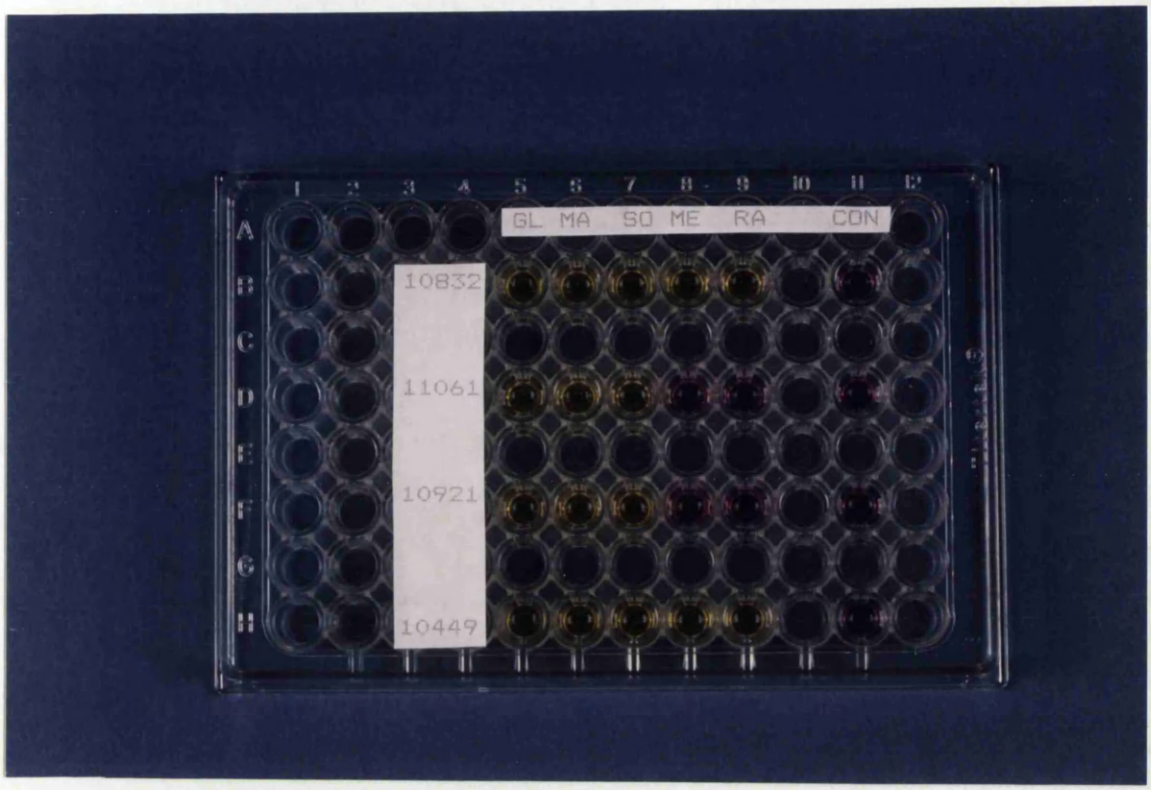
Atypical colonies which failed to produce profiles consistent with either mutans streptococci or Lactobacillus species were discarded.

2.2.4 Storage of bacteria.

After isolation, enumeration and identification, bacterial isolates were stored by freeze-drying and on Protect beads (vide infra). Thus a bank of organisms, isolated from children for whom caries prevalence and incremental data would be available, was created. Mutans streptococci strains were stored both in the freeze-dried and frozen state on Protect beads, whilst Lactobacillus strains were stored only on Protect beads.

Freeze Drying

A heavy suspension of each isolate was prepared in five millilitres of anaerobic blood broth (Gibco, Paisley,



GL = Glucose MA = Mannitol

SO = Sorbitol ME = Melibiose

RA = Raffinose CON = Control

NCTC 10832 = Serotype c

NCTC 11061 = Serotype g

NCTC 10921 = Serotype d

NCTC 10449 = Serotype c

Figure 2.4. Microtitre plate showing fermentation reactions of mutans streptococci.

Scotland) supplemented with 10% horse serum (Gibco, Paisley, Scotland) by transfer of the growth from a heavily inoculated blood agar plate with a sterile cotton swab. By means of a sterile Pasteur pipette (Alpha Laboratories, Hampshire, England), 0.5 ml of this suspension was transferred to a sterile, pre-labelled glass ampoule, which was then lightly re-plugged with a sterile cotton wool pledget.

The sample was then freeze-dried using an Edwards EF4 Modulto freeze-drier (Edwards High Vacuum, Crawley, England) under a vacuum of 1.3×10^{-1} mbar, by following the manufacturer's instructions. The vials were subsequently constricted with an Edwards ampoule constrictor and transferred to the secondary drying head on the freeze-drier. When a vacuum of 0.5×10^{-1} mbar had been achieved, the vials were sealed using an Edwards Flamemaster hand-torch. The vials were then stored in labelled racks at room temperature.

Protect Beads

An alternative method to preserve bacteria is to coat plastic beads with the organisms and then store them at -70°C (Protect Bacterial Preservers, Technical Service Consultants Ltd. Bury, England). Beads were supplied in plastic vials containing approximately 30 beads in a sterile medium of nutrient broth supplemented with 10% glycerol. A suspension of organisms to be stored was

prepared by transfer by a sterile cotton swab, from a blood agar plate supporting a heavy growth, to the vial containing the beads. The vial was shaken to thoroughly coat the beads and excess suspension medium removed with a sterile Pasteur pipette prior to freezing. The isolates were stored in duplicate at either -70°C or -30°C in freezers with alternative power supplies (Figure 2.5). The conventional method to preserve microorganisms is by freeze drying, but this relatively complex procedure is expensive in terms of equipment and time. Protect beads offer a cheaper, simple and quicker alternative but have the disadvantage that the beads are stored in a frozen state and may thus be lost due to a mechanical or electrical breakdown. Thus in addition to freeze-drying the samples were stored in duplicate in separate freezers.

2.2.5 Retrieval of Bacteria.

When cultures were required, freeze-dried ampoules were opened aseptically and the cells rehydrated with anaerobic blood broth (Gibco, Paisley, Scotland). The contents were then inoculated to blood agar plates and grown for 48 hours at 37°C in an atmosphere of air and 5% CO_2.

Isolates were recovered from the Protect beads by the aseptic transfer of one frozen bead to a blood agar plate. A sterile wire loop was then used to roll the bead over the surface of the plate, to ensure adequate

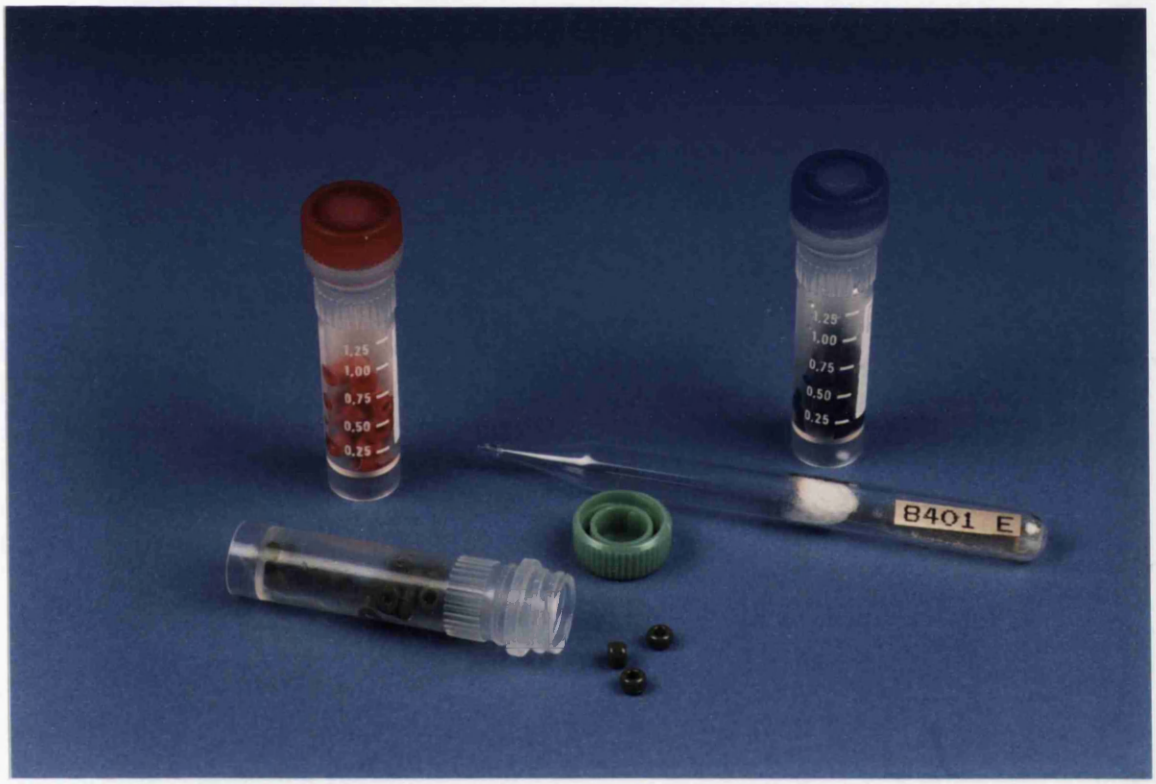


Figure 2.5. Protect beads and freeze dried ampoule.

inoculation. The plate was incubated at 37°C in an atmosphere of air and 5% CO_2 for 48 hours.

On retrieval from storage and prior to experimentation, the identity of randomly selected representative isolates was reconfirmed by means of colonial morphology, Gram staining and the biochemical tests as described in Section 2.2.3.

2.3 Isotachophoresis.

2.3.1 Introduction.

This section describes the technique of isotachophoresis, which was used throughout the study to assay acid anion production. Before a detailed explanation of the technique is undertaken (Sections 2.3.2 - 2.3.6) the theoretical basis of isotachophoresis is described.

In contrast to other electrophoretic techniques which are based on ions moving at different velocities, in isotachophoresis, ions possess the same migration velocity.

During migration in an electric field, ions of the same charge display different mobilities and will, at equilibrium, separate into distinct zones. Therefore if an electrolyte of intermediate mobility is introduced to a system containing two electrolytes, one of high mobility and the other low mobility (leading and terminating electrolytes), at equilibrium, three zones will result. The length of the zone is dependant on the

amount of electrolyte present.

By altering the pH, ionic strength or mobility of the leading and terminating electrolytes, in relation to the pH, composition and load of the sample ions, the separation of the ions into distinct zones may be optimised (Andrews 1988). Zones may be detected on the basis of ultra-violet absorbance, temperature, conductivity, or potential gradient changes at zone boundaries.

The use of isotachophoresis to determine acid anions, produced by bacteria during fermentation reactions, has been reported by Geddes and Weetman (1981), and a modification of this method was used in this study.

2.3.2 Apparatus.

Acid anions were detected using an LKB 2127 Tachophor (LKB Instruments, Broma, Sweden) fitted with a conductivity detection system (Figure 2.6). Separation was achieved in a 610 mm two-turn teflon capillary tube (internal diameter 0.5 mm) maintained at a constant 12°C. The leading electrolyte was 5 mM hydrochloric acid (BDH Chemicals Ltd., England) adjusted to pH 4.0 by the addition of beta-alanine (BDH Chemicals Ltd., England) which acted as the counter-ion thereby increasing the resolution of separation per unit length of tube. Hydroxypropyl-methylcellulose (Sigma, England), was added at a concentration of 0.1% w/v to the leading electrolyte, which by acting as a viscosity increasing

Figure 2.6. Isotachophor.

agent helps to increase the sharpness of zone boundaries by reducing electroendosmosis (Shah et al., 1985).

The terminating electrolyte was saturated, 5 mM n-octanoic acid (BDH Chemicals Ltd., England). All solutions were prepared with double-distilled deionized water.

A detection current of 150 μA was used for the first five minutes of separation and then reduced to 50 μA, with equilibrium and detection being achieved after a further 10 - 15 minutes, depending on the sample load. Under the conditions described it was possible to identify and quantify formate, pyruvate, phosphate, lactate, succinate, acetate and propionate.

2.3.3 Sample loading.

Via a three way injection port, leading and terminating electrolytes were introduced to the detection chamber, the latter having first been flushed with leading electrolyte. An appropriate volume (0.5-3 μl) of the specimen under investigation was withdrawn from the storage vial with a Hamilton Microsyringe (Scientific Glass Engineering, Ringwood, Australia), care being taken to ensure that the contents were mixed thoroughly, and that the vial remained uncapped for the minimum time possible. The sample was then injected into the tachophor via the designated port in the detection chamber.

2.3.4. Sample Identification.

When the separation of the acid anions was complete and equilibrium achieved, a trace (or isotachopherogram), was produced by a chart recorder linked to the detector. Shown in Figure 2.7 is a schematic illustration of an isotachopherogram.

Qualitative determination of the acid anions present in the unknown sample was determined by comparing the relative step heights of each component in the isotachopherogram with the step heights produced by a standard solution. The heights of each anion above the leading electrolyte is constant for given operational conditions. Furthermore, "priming" the sample with a known acid anion was used as an adjunct to identification of unknown ionic species when the conductivity detector indicated a lengthening of the particular zone in the isotachopherogram (Andrews 1988).

Quantitative determination of acid anions in an unknown sample was determined by measuring the zone lengths (under times eight magnification) produced by the specific acid anions on the isotachopherogram. The acid anion content was then calculated using the following formulae which permits the conversion of the zone length to acid anion concentration.

$$\frac{[(\text{Zone length} - c) \times 1/m]}{\text{Volume injected in tachophor}} \qquad \text{Eq. (1)}$$

$$\frac{\text{Volume of substrate}}{\text{Wet Weight of Bacteria}} \qquad \text{Eq. (2)}$$

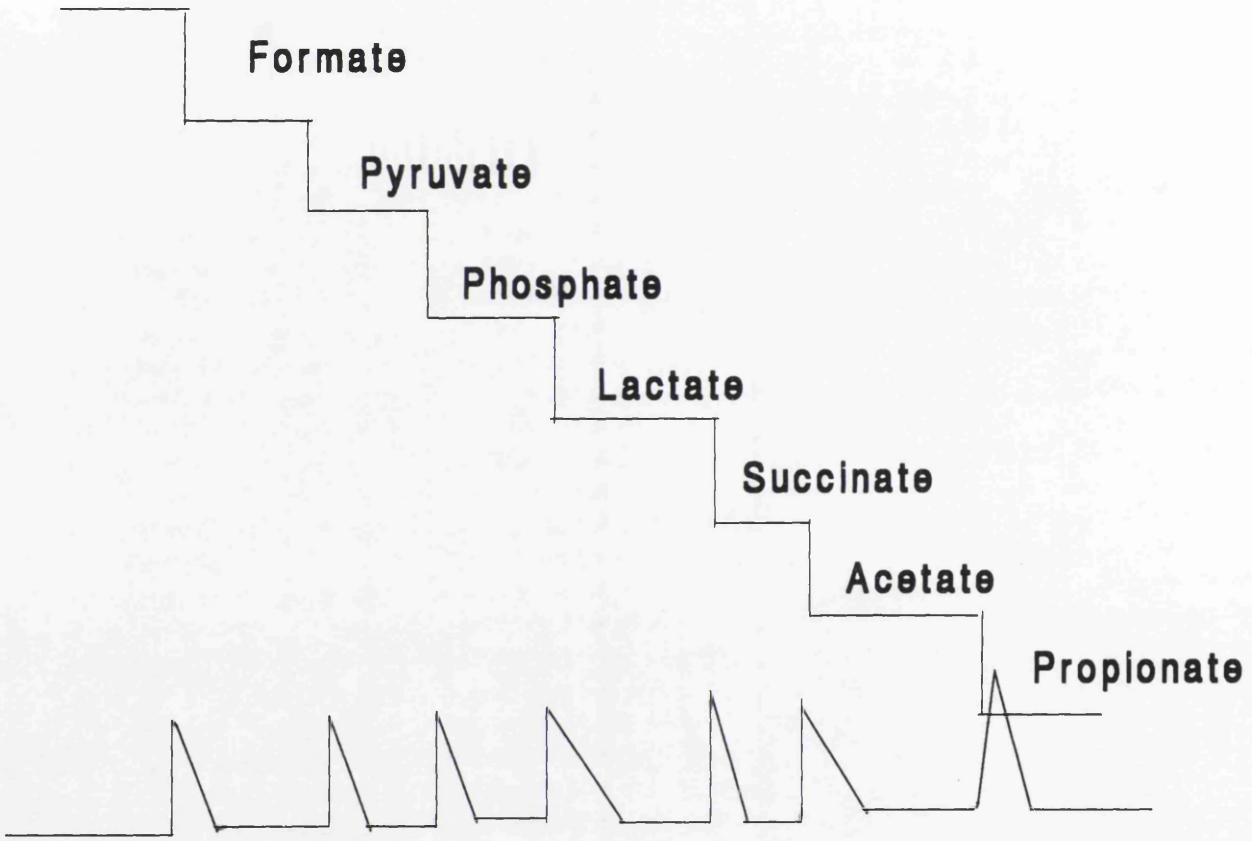


Figure 2.7. Diagrammatic representation of isotachopherogram.

Where

Zone length = distance produced by specific acid anion on isotachopherogram in millimetres.

c = intercept of calibration curve.

m = gradient of calibration curve / molarity of standard acid solution.

Volume of substrate = volume of sucrose in vial during assessment of cariogenic potential in microlitres.

Wet weight of bacteria = weight of bacteria in vial during assessment of cariogenic potential in milligrams.

As the standard acid solution contained millimolar concentrations of the acid anions and test samples were injected in microlitre volumes, Eq. 1 represents the concentration of acid anion in nanomoles per microlitre. The product of Eq. 1 and Eq. 2 represents the concentration, in nanomoles per milligram wet weight of bacteria, of the supernatant of the vial following incubation to assess cariogenic potential.

2.3.5 Determination of constants.

The constants c and m were determined as follows. A standard solution containing 5 mM of acetic, formic, lactic, phosphoric, pyruvic, propionic and succinic acids (BDH Chemicals Ltd., England) was prepared as in Appendix B.

Samples of the standard acid solution were injected in volumes of 0.5-3 μl and the zone lengths recorded on the isotachopherogram measured with a hand lens (Leitz, Wetzlar, Germany) under times eight magnification. Zone lengths produced by each of the specific anions were plotted against the amount of sample injected, Table 2.1. From these plots the correlation of zone length to volume of standard acid solution injected was determined. The intercept c, and slope m, of the curves were used to determine acid anion content using the formulae above. The constants c and m, were specific for a given batch of leading and terminating electrolytes, and were therefore recalculated when fresh electrolytes were prepared (see Section 2.5)

Data in Table 2.2, calculated using information from Table 2.1, illustrate typical values for c and m, and represent the constants used to calculate acid anion concentrations in the following Section.

2.3.6 Calculation of acid anion concentration.

As an example, this Section illustrates the calculation of acid anion concentration following the analysis of 1 μl of the supernatant of a vial in which 40.6 mg wet weight of a slurry of S. mutans had been incubated with 400 μl of a 5% sucrose solution, (see Section 3.4).

From the isotachopherogram produced, zone lengths were calculated as recorded in Table 2.3. By substitution of the above parameters and the constants determined in

vol	Form	Pyru	Phos	Lact	Succ	Acet	Prop
0.5	4.80	1.65	6.15	4.35	6.05	5.05	4.15
0.5	4.65	1.70	6.30	4.40	6.35	5.00	5.10
1.0	8.55	3.00	11.50	8.30	12.60	9.85	8.65
1.0	8.45	3.05	11.70	8.40	12.85	9.50	8.45
1.5	13.60	4.40	18.05	12.60	19.35	14.25	12.10
1.5	12.00	4.35	16.60	12.25	19.50	13.55	12.45
2.0	15.95	5.60	22.00	16.50	26.20	18.70	16.50
2.0	15.45	5.50	21.20	15.75	24.80	17.65	14.35
2.5	19.75	7.00	27.40	20.55	32.45	21.35	18.25
2.5	19.70	7.00	26.70	20.40	32.35	22.20	18.65
3.0	23.45	8.30	31.65	24.40	38.10	26.05	22.30
3.0	23.65	8.45	32.30	24.70	39.00	26.40	22.00

Vol.= volume (µl)

Form.= Formate
Phos.= Phosphate
Succ.= Succinate
Prop.= Propionate
Pyru.= Pyruvate
Lact.= Lactate
Acet.= Acetate

all length of trace (mm)

Table 2.1. Relationship between volume of standard acid solution injected and length of trace produced on isotachopherogram.

Acids	mM	r	c	m	1/m
Formate	4.99	0.9977	1.10	1.4940	0.6693
Pyruvate	5.10	0.9996	0.34	0.5218	1.9164
Phosphate	4.99	0.9984	1.36	2.0512	0.4875
Lactate	4.99	0.9995	0.29	1.6121	0.6203
Succinate	5.02	0.9994	-0.22	2.5802	0.3876
Acetate	5.00	0.9984	1.14	1.6751	0.5970
Propionate	4.99	0.9955	1.50	1.3831	0.7230

mM = true molarity of acid in standard acid solution.

r = correlation coefficient

c = intercept.

m = gradient of line divided by molarity of standard acid solution.

1/m = reciprocal of gradient / molarity of standard acid solution.

Table 2.2. Constants calculated from plots of volume of standard acid solution injected, against distance of tracing on isotachopherogram.

Acid anion	Zone Length (mm)	c	1/m	Acid anion (nmol/mg wet weight)
Formate	0.0[a]	1.10	0.6693	0.00
Pyruvate	2.8	0.34	1.9164	46.44
Phosphate	24.7[b]	1.36	0.4875	112.01
Lactate	40.8	0.29	0.6203	247.57
Succinate	1.4	-0.22	0.3876	5.34
Acetate	5.9	1.14	0.5970	27.99
Propionate	1.2[a]	1.50	0.7230	0.00

[a] When the distance on the isotachopherogram is less than c, acid anion content is regarded as zero.

[b] Whilst phosphate is detected during isotachophoresis, throughout the study this acid anion is excluded from the reported total acid anion concentration for the reasons discussed in Section 6.3.4.

Table 2.3. Calculation of acid anion concentration from zone length on isotachopherogram.

Table 2.2 to the conversion formulae, acid anion concentrations can be calculated and are reported in Table 2.3.

It is obviously important that in utilising any analytical technique that information is obtained pertaining to the precision of the method. As such data relating to isotachophoretic analysis were not readily available, the following studies, (Sections 2.4 & 2.5) were conducted.

2.4 Variation in isotachophoretic analysis.

2.4.1 Introduction.

The aim of this study was to investigate the variability in the determination of the acid anion content of six samples analysed on three separate occasions by isotachophoresis.

2.4.2 Methods.

As described in Section 3.4, six strains of S. mutans were prepared and incubated in the presence of a 5% sucrose solution and powdered hydroxyapatite for five hours. The supernatants, labelled A-F, resulting from these incubations were stored at -30°C, and the acid anion content of each assessed by isotachophoresis on three separate occasions over a one week period.

Statistical analysis.

Throughout this thesis statistical analyses were conducted using the Minitab Statistical Software Package (Minitab Incorporated, Pennsylvania, U.S.A.) on an IBM compatible personal computer and the University of Glasgow mainframe computer. In addition, multiple range analyses were performed by the Statgraphics Statistical Package (Statistical Graphics Corporation, Maryland, U.S.A.).

In this study, variation in repeat analysis was determined by the coefficient of variation, which is a measure of relative variation represented by division of the standard deviation by the mean, the result being expressed as a percentage (Haber & Runyon 1980).

2.4.3 Results.

The degree of variability observed when samples A-F were analysed on each of three occasions is indicated in Table 2.4. The coefficient of variation in individual acid anions ranged from 0% - 173.3%, whilst for total acid anions, values ranged from 5.0% to 12.4% with a mean of 7.8%. Greatest variability on repeat analysis was in the acid anions present in smallest quantities, namely formate, pyruvate and succinate. These acids are most likely to be influenced by the degree of separation, as loss of sharpness at zone boundaries increases the difficulty of identifying accurately and measuring zone

Sample A

Acid anion	Acid anion (nmol/mg wet weight bacteria)		
	Mean	S.D.	Co. Var.
Formate	2.10	3.64	173.3
Pyruvate	26.98	5.69	21.1
Lactate	710.74	99.07	13.9
Succinate	7.16	1.73	24.2
Acetate	84.71	11.98	14.1
Propionate	0.00	0.00	0.0
Total Acids	831.69	103.03	12.4

Sample B

Acid anion	Acid anion (nmol/mg wet weight bacteria)		
	Mean	S.D.	Co. Var.
Formate	0.00	0.00	0.0
Pyruvate	22.52	20.79	92.3
Lactate	283.83	34.41	12.1
Succinate	4.70	0.79	16.8
Acetate	31.72	3.35	10.6
Propionate	0.00	0.00	0.0
Total Acids	342.77	17.13	5.0

Sample C

Acid anion	Acid anion (nmol/mg wet weight bacteria)		
	Mean	S.D.	Co. Var.
Formate	4.35	4.14	95.2
Pyruvate	14.06	3.51	25.0
Lactate	560.45	36.18	6.4
Succinate	9.19	5.30	57.7
Acetate	84.71	11.98	14.1
Propionate	0.00	0.00	0.0
Total Acids	669.76	44.07	6.6

Table 2.4. (i) Variation in acid anion concentration on repeated analysis (n=3)

Sample D

Acid anion	Acid anion (nmol/mg wet weight bacteria)		
	Mean	S.D.	Co. Var.
Formate	5.51	4.78	86.7
Pyruvate	12.82	9.28	72.4
Lactate	315.57	12.69	4.0
Succinate	10.31	8.55	82.9
Acetate	67.84	4.27	6.3
Propionate	0.00	0.00	0.0
Total Acids	**412.05**	**23.64**	**5.7**

Sample E

Acid anion	Acid anion (nmol/mg wet weight bacteria)		
	Mean	S.D.	Co. Var.
Formate	0.00	0.00	0.0
Pyruvate	21.42	17.25	80.5
Lactate	296.26	59.11	20.0
Succinate	5.00	0.38	7.6
Acetate	31.16	17.69	56.8
Propionate	0.00	0.00	0.0
Total Acids	**353.84**	**24.81**	**7.0**

Sample F

Acid anion	Acid anion (nmol/mg wet weight bacteria)		
	Mean	S.D.	Co. Var.
Formate	0.00	0.00	0.0
Pyruvate	30.67	10.09	32.9
Lactate	266.94	28.26	10.6
Succinate	6.21	0.60	9.7
Acetate	37.29	3.66	9.8
Propionate	0.00	0.00	0.0
Total Acids	**341.11**	**34.54**	**10.1**

S.D. = Standard Deviation Co. Var.=Coefficient of Variation

Table 2.4. (continued) Variation in acid anion concentration on repeated analysis (n=3).

lengths of about 1-2 millimetres. Additionally, the large variation was in part due to these anions, on occasion being detected on only two of the three analyses. These acids, however, comprise less than 10% of the total acid production. In the case of the major acid anions present, namely lactate and acetate, coefficients of variation were respectively 11.2% and 18.6%.

2.4.4 Discussion.

An obvious potential source of variability in results was the very small volume, (1µl), of sample injected into the isotachophor. This was achieved with a Hamilton microsyringe to which was attached a calliper to ensure uniform delivery of the sample volume. Whilst it would have been desirable to increase the sample volume, thereby reducing variability due to entrapment of air bubbles or other errors in sample delivery, an increased volume usually leads to deterioration in the quality of separation. In order to overcome this problem, the sample was diluted prior to increasing the volume injected, in an initial experiment. However this resulted in failure to detect those acids present in small quantities.

Other possible causes of error were changes during storage of the vial, or loss of vial contents due to evaporation during sampling. Therefore care was taken to

ensure that samples were thoroughly defrosted and well mixed prior to analysis, and that the storage vial remained uncapped for the minimum time possible. Whilst samples were stored frozen between analyses the small volumes available precluded aliquoting the samples into individual portions prior to repeat analysis.

From a survey of the literature, it is difficult to establish an average value for variation in analytical techniques. This is obviously dependant on the sensitivity of the technique being considered. In the present study a mean variation of 7.8% in total acid anion analysis was observed. The clear advantages of isotachophoresis over other techniques of acid anion identification and quantification, (in particular the ability to analyse small volumes), confirmed the initial decision to use this technique for acid anion analysis throughout the study, with the additional precautions described in Section 2.5.4.

2.5 Influence of leading and terminating electrolytes on isotachophoretic analysis.

2.5.1 Introduction.

The calculation of acid anion concentration (Section 2.3.6) and the reproducability study (Section 2.4), were conducted using one batch of leading and terminating electrolytes. As previously intimated, the constants c

and m are specific to each electrolyte batch and were recalculated as described (Section 2.3.5), when new electrolytes were made up. This investigation aimed to study the effect of different batches of leading and terminating electrolyte on isotachophoresis reproducability, as this aspect of isotachophoretic analysis has not previously been reported.

2.5.2 Methods.

Samples (A-F), previously analysed in Section 2.4, were examined on a further six occasions using two additional batches of leading and terminating electrolytes. Each sample was therefore analysed nine times in total, three times with each of three electrolytes (Batches I-III). Samples were analysed using a given batch of electrolytes over a one week period, with an interval of approximately three months between analysis with different batches of electrolytes.

2.5.3 Results.

The effect of different electrolytes on the total zone length on the isotachopherogram is reported in Table 2.5. The effect of electrolyte batch on reproducability, showed that the coefficient of variation for total zone length ranged from 0.5% to 12.4%. The mean coefficient of variation for each of the electrolyte batches I, II and III was 7.3%, 7.3% and 4.5% respectively. Analysis of variance failed to show any significant differences in

Sample	Total Zone Length (mm)		
	Electrolyte Batch		
	I	II	III
	Mean[a] (S.D.)	Mean[a] (S.D.)	Mean[a] (S.D.)
A	114.6 (12.4)	91.2 (5.2)	90.8 (4.6)
B	56.0 (4.8)	53.5 (2.3)	58.6 (0.5)
C	113.7 (7.5)	114.5 (12.0)	102.7 (9.2)
D	60.1 (3.7)	62.1 (9.2)	54.5 (3.1)
E	61.9 (4.7)	64.8 (0.3)	61.0 (3.1)
F	47.7 (1.9)	53.0 (4.2)	44.2 (0.8)

S.D. = Standard Deviation.
[a] = mean of three determinations

Table 2.5. Effect of leading and terminating electrolytes on total zone length (mm) on isotachopherogram.

Sample	F Value	P value
A	8.24	0.02
B	1.22	0.36
C	1.37	0.32
D	1.30	0.34
E	0.74	0.52
F	7.93	0.02

Degrees of Freedom = 2.6.

Table 2.6. Significance of differences in total zone length of repeat isotachophoretic analysis using three batches of electrolytes, each measured on three occasions, as assessed by analysis of variance.

variation between batches.

The effect of the different electrolyte batches on total zone length was assessed by analysis of variance and is reported in Table 2.6. In the case of samples A and F, significant differences in the zone length were observed ($p < 0.02$), when these samples were analysed by different batches of electrolyte.

2.5.4 Discussion.

The effect of electrolyte batch has not been reported previously. In the light of the findings in Table 2.6, care was taken throughout this study to ensure that all isotachophoretic analysis within a given experiment or set of experiments, were conducted using one batch of leading and terminating electrolytes.

Andrews (1988) stated that more factors need to be optimised in order to obtain satisfactory results in isotachophoresis than in any other electrophoretic method, a fact which has lead to under-utilisation of the technique. However, particular advantages of the method are relatively rapid analysis times and good resolution, combined with easy quantitative measurements.

Shah et al., (1985), concluded that this technique had considerable advantages over gas liquid chromatography for the detection of short chain carboxylic acids, as the sample does not require pretreatment and loss of acids

due to interaction with the column or "ghosting" does not occur (Geddes & Gilmour, 1970).

With the appropriate precautions described in Section 2.4.4 and above, it was decided that throughout this thesis acid anion analysis would be conducted using isotachophoresis.

2.6. Calcium assay.

2.6.1 Introduction.

Calcium release from mineral substrate was determined spectrophotometrically following complexation with o-cresolphthalein complexone (Sigma Chemical Company Ltd., Poole, England). In an alkaline medium (pH 10-12), calcium reacts with o-cresolphthalein complexone to form a purple coloured complex, with the intensity of the colour change being directly proportional to the amount of calcium in the sample (Figure 2.8). The method is accurate only in the range 1-15 mg/dl.

2.6.2 Method.

The kit was supplied as two reagents, the calcium binding reagent and the calcium buffer reagent. These were combined in equal quantities to form the working solution, then aliquoted in one millilitre amounts to disposable plastic micro-square cuvettes (Elkay Laboratory Products, Basingstoke, England).

Absorbances were read using a Pye Unicam SP8-100 VV/VIS Spectrophotometer (Pye-Unicam Ltd, Cambridge, England)

Figure 2.8. Complexation of o-cresolphthalein with decreasing concentrations of calcium.

with a 0.5 cm lightpath at 575 nm as maximum absorbance occurred under these conditions. The spectrophotometer was calibrated using blank micro-cuvettes containing one millilitre distilled water. Samples were then analysed as described below and calcium content determined by the following formula.

$$[(F_T - I) / (F_S - I)] \times 10 = \text{Calcium concentration mg/dl}$$

where

F_T = absorbance of the test or sample solution

F_S = absorbance of the standard solution

I = absorbance of the working solution

I was taken as the mean of the absorbance of six vials containing the working solution

F_S was taken as the mean of the absorbance of six vials to which had been added 10 µl of the standard calcium solution containing 10 mg/dl calcium. (Sigma Chemical Company Ltd., Poole, England).

A Hamilton Microsyringe was used to add 10 µl of the sample being assayed to a microcuvette containing one millilitre of the working solution. The cuvette was lightly agitated to ensure through mixing of the contents before the absorbance (F_T) was measured. Throughout the study, calcium assays are reported as the mean of three

determinations, the calcium concentrations being measured on three separate occasions with different preparations of the working solution.

As absorbance was linear only to 15 mg/dl, samples containing greater concentrations were diluted as appropriate and allowance made accordingly when the calcium content was calculated.

The standard calcium solution contained 10 mg/dl, and results obtained using the above formula were expressed in mg/dl and were subsequently converted to millimoles per litre.

2.6.3 Calculation of calcium content.

This section illustrates the calculation of calcium content in the supernatant of a vial incubated as described in Section (3.4).

The first stage involved determination of the constants I and F_s, for each of three preparations of the working solution identified as (i), (ii) and (iii). These were taken as the mean absorbance of six cuvettes containing the working and standard solutions, and are reported in Table 2.7.

The second stage involved determination of the calcium content represented by the absorbance F_T. As the sample contained more than 15 mg/dl of calcium, a 1:10 dilution of the sample was measured. The absorbances F_T, determined using each of the three preparations of the working

Working Solution (i)	Standard Solution
0.458	0.905
0.460	0.880
0.460	0.863
0.469	0.874
0.472	0.860
0.457	0.864
I = 0.462	F_s = 0.874

Working Solution (ii)	Standard Solution
0.443	0.896
0.458	0.897
0.459	0.898
0.447	0.890
0.451	0.879
0.450	0.857
I = 0.451	F_s = 0.886

Working Solution (iii)	Standard Solution
0.449	0.867
0.450	0.863
0.441	0.865
0.442	0.875
0.441	0.865
0.471	0.873
I = 0.449	F_s = 0.868

I = absorbance of working solution.
F_s = absorbance of standard solution.

Table 2.7. Calculation of the constants I and F_s using three different preparations (i-iii) of the working solution.

	WORKING SOLUTION		
	(i)	(ii)	(iii)
F_T	0.618	0.614	0.613
Calcium (mg/dl)	37.71	37.47	39.14
Calcium (mmol/L)	9.41	9.35	9.76

Table 2.8. Calculation of calcium concentration using three preparations of working solution.

solution, are as reported in Table 2.8. By substitution of the appropriate values of F_T, F_s and I in the formula described in Section 2.6.2, and correcting for the dilution factor, the calcium content in mg/dl and mmol/L can be calculated as in Table 2.8.

2.7 Variation in calcium assay.

2.7.1 Introduction.

The aim of this study was to investigate the variation in calcium assay of six samples (A-F), when determined using different preparations of the working solution. The samples used were those employed in Section 2.4 to study variation in isotachophoretic analysis. Samples were assayed three times on each of three occasions, using three different preparations of the working solution (i, ii and iii) described in Section 2.6.3.

2.7.2 Methods.

Calcium content was assayed as described in Section 2.6.2., and statistical analysis conducted as described in Section 2.4.2.

2.7.3 Results.

The mean, standard deviation and coefficient of variation of the repeat analysis are reported in Table 2.9. The coefficient of variation ranged from 2.47% to 5.30% with a mean of 4.12%, which compares favourably with the variation encountered in isotachophoretic analysis.

Sample	Calcium concentration (mmol/l)		
	Mean[a]	S.D.	Co. Var.
A	14.24	0.57	4.03
B	7.73	0.47	5.30
C	14.53	0.71	4.90
D	7.42	0.81	2.47
E	7.58	0.32	4.22
F	5.18	0.24	4.36

S.D. = Standard deviation.

Co. Var.= Coefficient of Variation.

[a] = samples were assayed three times on each of three occasions using three different preparations of the working solution.

Table 2.9. Variation in calcium analysis on three separate analyses.

2.7.4 Discussion.

By calculating mean absorbances of working and standard solutions (F_s and I), and using three batches of working solution, the potential error introduced by, (a) injection technique, (b) variation in absorbance of the disposable cuvettes and (c) batch variations in the working solution, were minimised. The variation observed in repeat analyses was judged to be within acceptable limits, and it was therefore decided that this technique would be used throughout this thesis to assay calcium release during the determination of cariogenic potential.

2.8 Comparison of calcium analysis by complexation with O - cresolphthalein complexone and by atomic adsorption spectroscopy.

2.8.1 Introduction.

Calcium analysis by complexation with o-cresolphthalein was designed primarily for the clinical determination of calcium in serum. As this technique has not been widely reported in the literature, this study aimed to compare the calcium content of twelve vials containing a range of calcium concentrations as assayed by o-cresolphthalein and by atomic adsorption spectroscopy.

2.8.2 Method.

A series of twelve vials containing a range of calcium concentrations obtained during assays of <u>in vitro</u>

cariogenic potential, (Section 6.2), and two control samples containing double distilled deionised water, were used in this study. Calcium content following complexation with o-cresolphthalein was determined as described in Section 2.6.2. The samples were then labelled in random order, frozen and transported overnight to the laboratory of Dr. D.J. Page, Unilever Research, Portsunlight, England. Samples were then assayed by atomic absorption spectroscopy without prior knowledge of the results obtained using the colourimetric method.

2.8.3 Results.

Data in Table 2.10 compare calcium assay results following complexation with o-cresolphthalein and assay by atomic adsorption spectroscopy. Values determined by o-cresolphthalein represent the mean of three estimations, whilst the atomic adsorption assays are the mean of two determinations. Values obtained ranged from 0 - 18.6 mmol/l and 0 - 15.4 mmol/l with the o-cresolphthalein and atomic adsorption respectively. The values obtained with the atomic adsorption were, in general, 2-3 mmol/L lower than those detected by the colourimetric test. A significant correlation between values as assessed by the two methods was observed, with a highly significant ($p<0.001$) correlation coefficient of 0.958 being obtained, Figure 2.9.

Sample	Calcium (mmol/l)	
	o-cresolphthalein[a]	Atomic adsorption[b]
1	0.00	0.00
2	0.00	0.01
3	3.50	1.20
4	7.80	5.01
5	7.87	6.00
6	7.99	3.80
7	8.09	5.80
8	8.30	5.20
9	12.05	5.10
10	12.18	9.70
11	13.26	9.60
12	14.15	11.20
13	18.43	14.60
14	18.60	15.40
	$r = 0.958$ $p < 0.001$	

[a] = mean of three determinations

[b] = mean of two determinations

Table 2.10. Comparison of calcium determination assayed by complexation with o-cresolphthalein and by atomic adsorption spectroscopy.

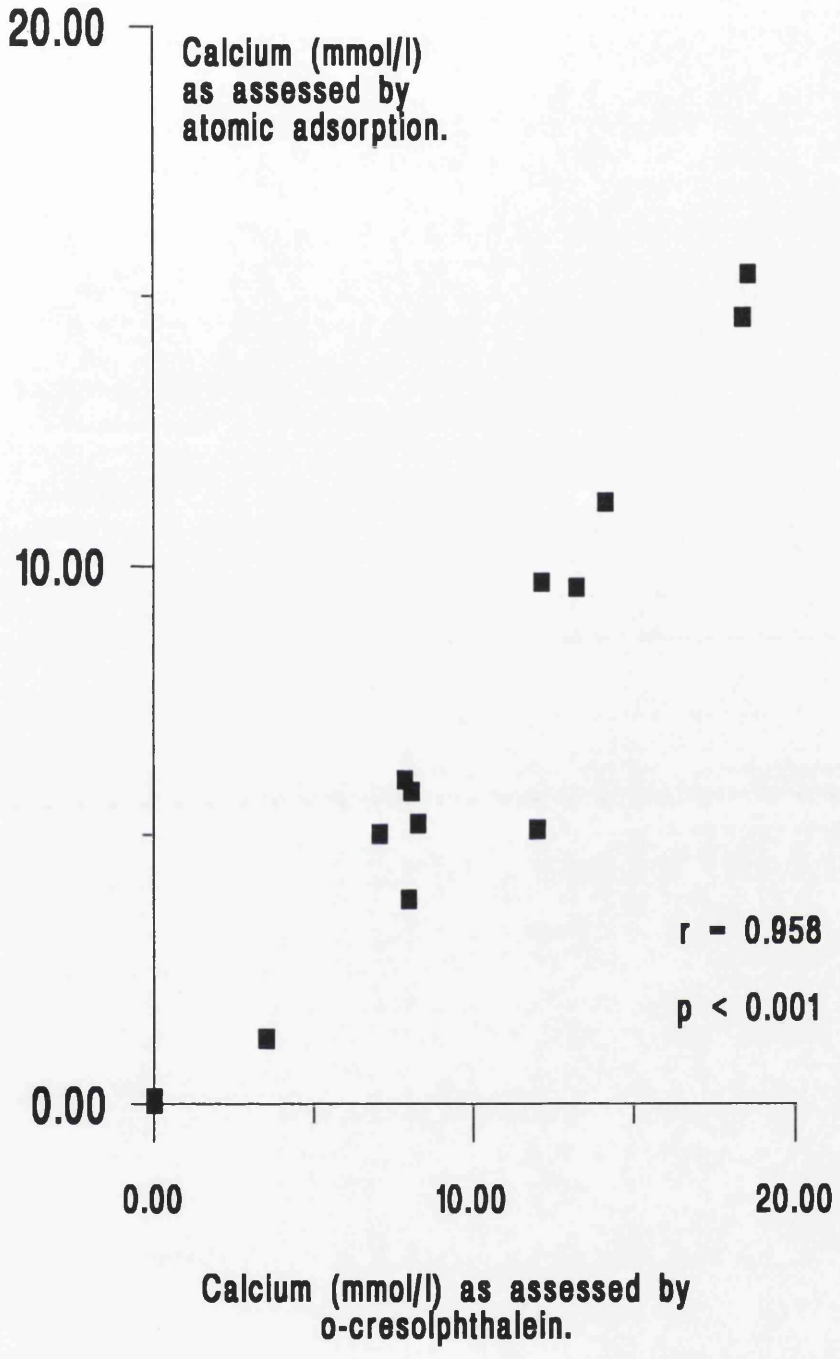


Figure 2.9. Comparison of calcium determination by atomic adsorption spectroscopy and complexation with o-cresolphthalein.

2.8.4 Discussion.

From Table 2.10 and Figure 2.9 it can be seen that there was a significant correlation between calcium content of the vials as assessed by the methods described. However the values obtained by atomic adsorption were lower than those observed with the o-cresolphthalein. The latter combined only with ionised calcium and it is therefore surprising that the atomic adsorption should reveal lower levels. There are, however, a number of possible explanations for this disparity. Patel, Fox and Higuchi (1987) reported that phosphate may interfere with calcium determination by atomic adsorption and that this problem may be overcome by the precipitation of the phosphate by the addition of lanthium oxide. The failure to account for the presence of the phosphate in this experiment may explain the lower calcium determination by atomic adsorption. The total volume of the samples available for assay by atomic adsorption was about 300 µl, and as a volume of one millilitre was required for analysis, samples had therefore to be diluted. Hence it is possible the lower readings observed may be attributable to dilution effects.

From these investigations it was concluded that complexation with o-creslophthalein proved a suitable method for detection of calcium content of vial supernatants and was used throughout this study.

2.9 pH Measurement.

2.9.1 Materials.

A 220 pH meter (Ciba Corning Diagnostics Ltd., Suffolk, England) and a micro-combination electrode (Kent Industrial Measurements Ltd., Gloucestershire, England) were used to measure pH. The pH meter was calibrated daily in accordance with the manufacturer's instructions using standard buffer solutions of pH 4, pH 7, pH 9, (BDH Chemicals Ltd, Poole, England).

2.9.2 Method.

During assessment of cariogenic potential, the pH of the vial contents was measured by withdrawing the electrode from the storage buffer solution (pH7), rinsing it with sterile distilled water, drying it, and then immersing it in the vial for 10 seconds. The vial was lightly agitated throughout the period of measurement. The pH reading was recorded and the electrode was thoroughly rinsed with sterile distilled water before reimmersion in the storage buffer.

When the pH of more than one vial was to be recorded, the reading on the meter was allowed to return and stabilise at pH 7 before proceeding to the next vial.

CHAPTER 3

THE DISSOLUTION OF MINERAL SUBSTRATES IN THE DETERMINATION OF THE *IN VITRO* CARIOGENIC POTENTIAL OF *S. MUTANS*.

3.1 Introduction

This Chapter describes an *in vitro* test, which involves the dissolution of bovine enamel as a means of assaying the cariogenic potential of six strains of *S. mutans*. It then describes an investigation of alternative mineral substrates for the determination of cariogenic potential.

3.2 The dissolution of bovine enamel in the determination of *in vitro* cariogenic potential.

3.2.1 Introduction.

The dissolution of bovine enamel as a means of assaying the cariogenic potential of *S. mutans* has been reported by Macpherson, (Macpherson, 1988; Macpherson *et al.*, 1992). That study investigated the cariogenic potential of six strains of *S. mutans* isolated from six adult volunteers. It was reported that when a slurry of each strain was incubated in the presence of an enamel slab, the fall in pH, acid anion production and calcium release showed significant correlations with the DMFS of the individuals from whom the strains had been originally isolated. Furthermore, the laboratory results correlated significantly with the *in vivo* experimental caries

experience of the individuals, as assessed by the demineralisation of enamel sections mounted on an intra-oral appliance.

3.2.2 Aim.

The aim of this experiment was to repeat the investigation of Macpherson (1988) and Macpherson et al., (1992).

3.2.3 Materials and methods.

Preparation of bacterial cells for incubation with bovine enamel.

The bacterial strains investigated by Macpherson (1988) were retrieved form freeze-dried ampoules as described in Section 2.2.5, and their identity confirmed as described in Section 2.2.3. The bacteria were then prepared for assay of cariogenic potential as detailed below, and as illustrated in Figure 3.1.

Using a sterile wire loop, heavy inocula of the strain under investigation were transferred to four Columbia blood agar plates, (see Appendix A), and streaked evenly over the entire surface to ensure uniform growth on each plate. These were then incubated for 24 hours in an atmosphere of air and 5% CO_2, at 37°C (LTE Qualitemp 80 MI, Laboratory Thermal Equipment, Oldham, England). Following incubation, sterile cotton swabs (Alpha

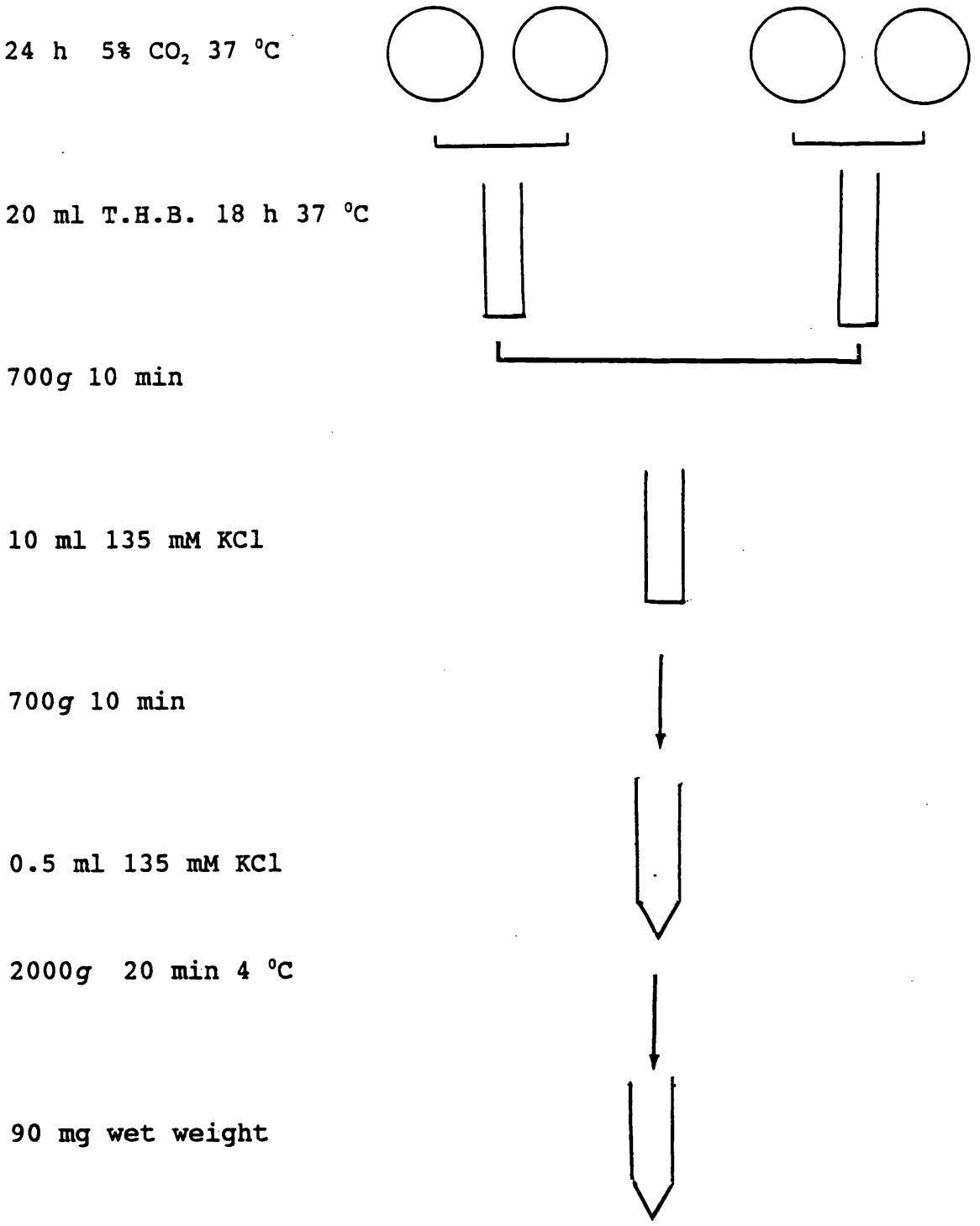
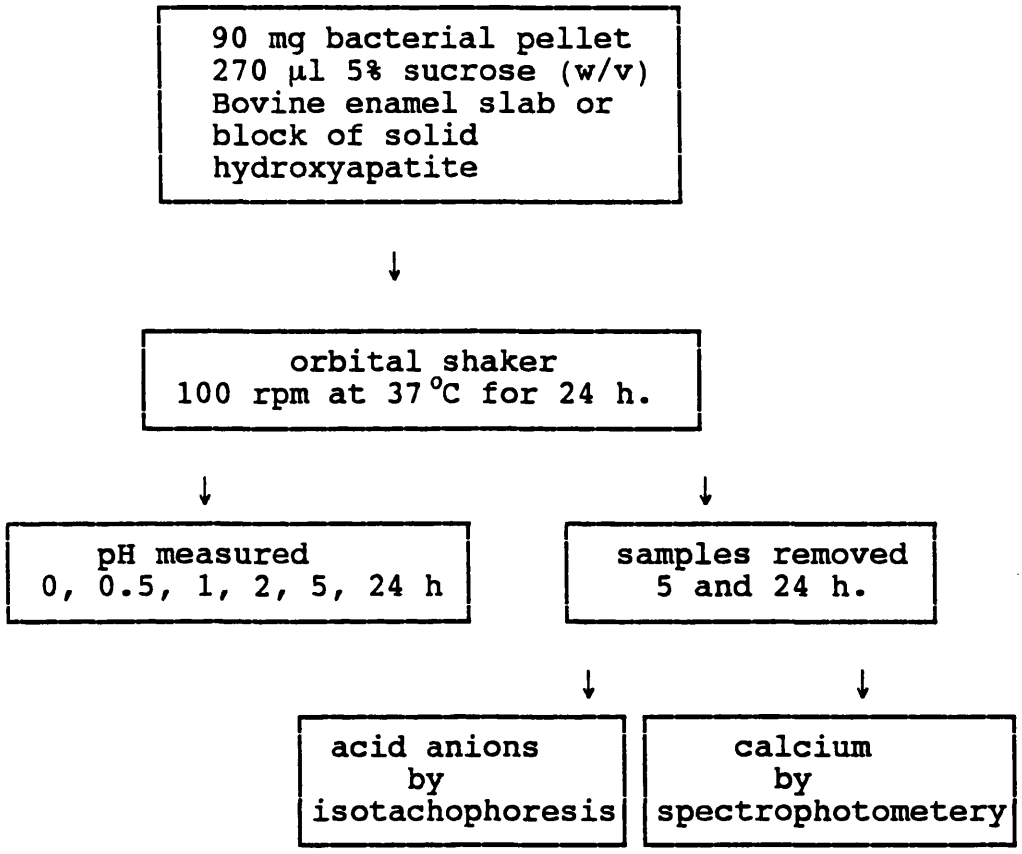


Figure 3.1. Diagrammatic representation of the preparation of bacterial cells for incubation with bovine enamel.

DAY 1



DAY 2

Enamel or solid hydroxyapatite slab rinsed and reincubated as above for a second 24 h period

Figure 3.2. In vitro assessment of cariogenic potential of S. mutans strains with bovine enamel.

Laboratories, Hampshire, England) were used to transfer aseptically, growth from each plate to Todd Hewitt Broth (Gibco, Paisley, Scotland). Bacterial cells from two plates were added to 20 ml of the broth in universal containers. Macpherson (1988) had used the glucose-containing chemically defined medium of Terleckyj, Willett and Shockman (1975). However, because of problems encountered in achieving complete dissolution of the constituents of this medium, it was decided that throughout this study, bacterial cells would be grown in Todd Hewitt Broth prepared from the same batch (lot number 27E2942).

After incubation for 18 hours at 37°C in an orbital shaker (Gallenkamp, England) at 100 rpm, the universal containers were centrifuged at 700 *g* in an MSE Centaur centrifuge (Fisons Instrumentation Services, Crawley, England), for ten minutes. The supernatants were discarded and the deposits resuspended in 10 ml 135 mM KCl (Marsh et al., 1982) and combined in one universal container. This was centrifuged once more at 700*g* for a further 10 minutes, when the supernatant was discarded and the pellet resuspended in 0.5 ml 135 mM KCl and transferred to a pre-weighed plastic vial (ref. no. 72.700 Sarstedt, West Germany).

This vial was centrifuged at 2000 *g* in an MSE HS18 centrifuge (Fisons Instrumentation Services, Crawley, England.) for 20 minutes at 4°C.

After centrifugation, the supernatant was removed with a

sterile needle and syringe (Becton Dickinson Labware, Oxford, England) and the pellet weighed on a Galaxy 160 balance (OHAUS Scale Corporation, New Jersey, USA). The weight of the pellet was adjusted with a sterile spatula so that 90 mg wet weight of bacteria remained in the vial. The resulting pellet was incubated with the bovine enamel to assess cariogenic potential.

Preparation of bovine enamel slabs.

With appropriate aseptic precautions, freshly extracted bovine incisors were washed in warm soapy water, then rinsed thoroughly. The labial aspect of the crowns were polished by bristle brush, mounted in the handpiece of a pendant motor (Milbro, Epson, England) operated at slow speed, using "fluoride free" pumice powder, and again thoroughly rinsed in cold running water. The labial surface of the enamel was examined (x15) with a stereo microscope (Nikon, Japan), to check for gross defects in enamel structure. Teeth found to be defective were discarded. The crown was sectioned from the root with a heavy duty carborundum cut-off disc (Chaperlin & Jacobs, London, England) at 18,000 r.p.m., without coolant, care being taken to avoid overheating the specimen.

Once any remnants of the dental pulp had been removed, a slab measuring approximately 5mm x 3mm x 1.5mm was prepared from the mid-buccal third of the crown. The enamel surface was then abraded by approximately 100 μm,

by hand-grinding with a slurry of silicon carbide, 600 grit (BDH Chemicals Ltd., England). Care was taken to ensure the enamel surface was at all times parallel to the grinding plate. The enamel slab was measured before, during and after grinding using an electronic micrometer (Mitutoyo, Japan), to confirm uniform abrasion.

The enamel slab was finally washed with deionised water and allowed to dry. Whilst being viewed under the stereo microscope, the slab was covered with an acid resistant varnish (Max Factor, London, England), to leave a window of exposed enamel surface measuring approximately 4 mm^2. The slab was then stored in deionised water at room temperature prior to incubation as detailed below.

Incubation of bacterial cells and bovine enamel.

The assay of cariogenic potential using bovine enamel as mineral substrate was undertaken as follows, and is described diagrammatically in Figure 3.2.

The assay was performed by the addition of 270 µl of a 5% sucrose solution (w/v) to the vial that contained the bacterial pellet prepared as described above (Figure 3.1). After agitation with a Spinmix (Gallenkamp, England) for 10 seconds to ensure mixing of vial contents, the bovine enamel slab was added and the vial agitated for a further 10 seconds. The pH of the vial

contents was then measured as described in Section 2.9.

The vial was then transferred to the orbital shaker, and incubated aerobically at 100 rpm and 37°C. The pH was again measured after 0.5, 1, 2, 5, and 24 hours' incubation. After 5 hours' incubation, 30 µl was removed and stored at -30°C prior to analysis of calcium and acid anion content. After 24 hours the bovine enamel slab was transferred to a freshly prepared bacterial pellet and the contents of the first vial stored at -30°C. The slab was then incubated at 37°C for a second period of 24 hours. During this time the pH was monitored, and samples removed and stored for analysis as described during the first 24 hours. At the end of this second incubation, the slab of enamel was withdrawn and the vial stored at -30°C.

Prior to analysis for acid anion and calcium content, the vials were removed from the freezer and allowed to thaw before centrifugation at 15000*g* for 20 min at 4°C. The supernatant was aspirated using a Hamilton microsyringe (Scientific Glass Engineering, Ringwood, Australia) and centrifuged for a second time in an identical fashion to ensure complete removal of all debris (Figure 3.3). The supernatant was transferred to a clean vial and again stored at -30 °C until analysis of acid anion content by isotachophoresis (see Section 2.3) and calcium content by complexation with o-cresolphthalein (see Section 2.6).

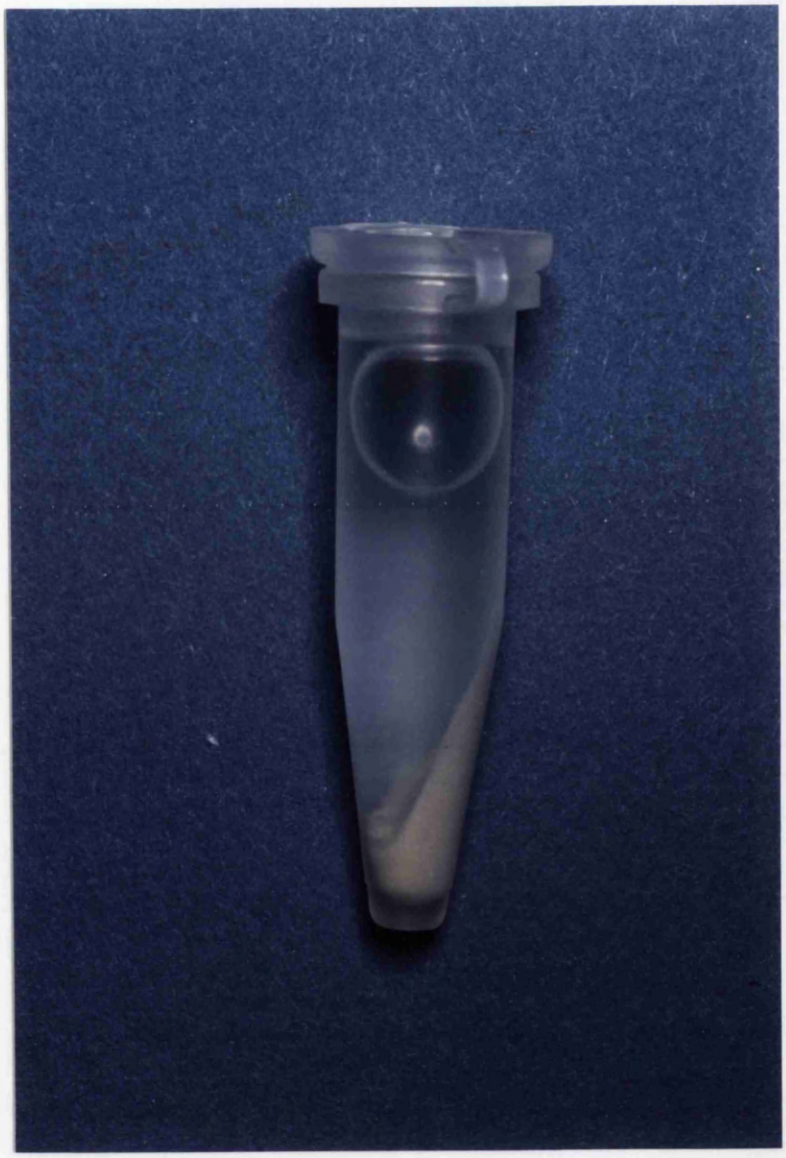


Figure 3.3. Vial containing supernatant and bacterial cell pellet following centrifugation.

Macpherson (1988) investigated six strains of S. mutans labelled A, B, D, E, F, G, and a Type Strain NCTC 10449 (labelled TC). With the exception of strain F, which was unavailable, these strains were reinvestigated in this study, strain G being redesignated strain C. Each strain was tested on three occasions, a total of six, 24 hour incubations. For technical reasons, data for the third incubation of strain B for the period 24-48 hours is unavailable.

Statistics

Statistical analysis was conducted as described in Section 2.4.2. Macpherson (1988) assessed the significance of differences between strains by multiple Students' t-tests. Recently, Brown and Swanson Beck (1990) have reported that such an approach to the comparison of more than two groups is inappropriate, and that such data are more correctly analysed by analysis of variance. The results of the present study were, for comparative purposes, analysed using both multiple Students' t-tests and analysis of variance.

3.2.4 Results

Calcium release during incubation with bovine enamel.

Calcium release after 5, 24 and 48 hours' incubation is reported in Table 3.1 and Figure 3.4. After the first five hour period of incubation, mean calcium release ranged from 0.23($\pm$0.12) mmol/mm^2 by strains B and C, to

Calcium (mmol/mm²).						
	5 hours		24 hours		48 hours	
Strain	mean[a]	(S.D.)	mean[a]	(S.D.)	mean[a]	(S.D.)
A	0.31	(0.15)	1.51	(0.36)	2.64	(0.44)
B	0.23	(0.12)	1.11	(0.47)	1.98[b]	
C	0.23	(0.10)	1.06	(0.32)	2.05	(0.58)
D	0.34	(0.08)	1.66	(0.08)	3.28	(0.29)
E	0.48	(0.20)	2.14	(0.38)	4.56	(0.36)
TC	0.29	(0.02)	1.74	(0.11)	4.27	(0.64)

Significance of differences between strains as assessed by analysis of variance.

Incubation period (hours)	5	24	48
Degrees of freedom	5, 12	5, 12	5, 11
F ratio	1.71	4.87	15.91
p value	0.206	0.01	0.001

Significant differences by 95% Scheffé tests.

After 24 hours	E v B and C.
After 48 hours	E v A, B and C. TC v A, B and C.

[a] = mean of three replicates
[b] = mean of two determinations (section 3.2.3)
S.D. = Standard Deviation
mmol/mm² = millimoles calcium per mm² exposed enamel.

Table 3.1. Calcium release after 5, 24 and 48 hour incubations with bovine enamel.

Strain	Calcium mmol/mm² bovine enamel.	
	mean[a]	(S.D.)
A	1.32	(0.35)
B	1.03	(0.47)[b]
C	1.03	(0.60)
D	1.64	(0.22)
E	2.28	(0.32)
TC	2.13	(0.61)

Significance of differences between strains as assessed by Students' t-tests.

E	v -A***	-B***	-C***	-D***
TC	v -A**	-B**	-C**	
D	v -B*	-C*		

*** = $p < 0.005$
** = $p < 0.01$
* = $p < 0.05$

Significance of differences between strains as assessed by analysis of variance.

Degrees of freedom = 5, 29
F ratio = 8.30
p < 0.001

Significant differences by 95% Scheffé tests.

E v A, B and C

TC v B and C.

[a] = mean of six replicates
[b] = mean and standard deviation of five replicates
S.D. = Standard Deviation
mmol/mm² = millimoles calcium per mm² exposed enamel.

Table 3.2. Calcium release during initial and subsequent 24 hour incubations with bovine enamel.

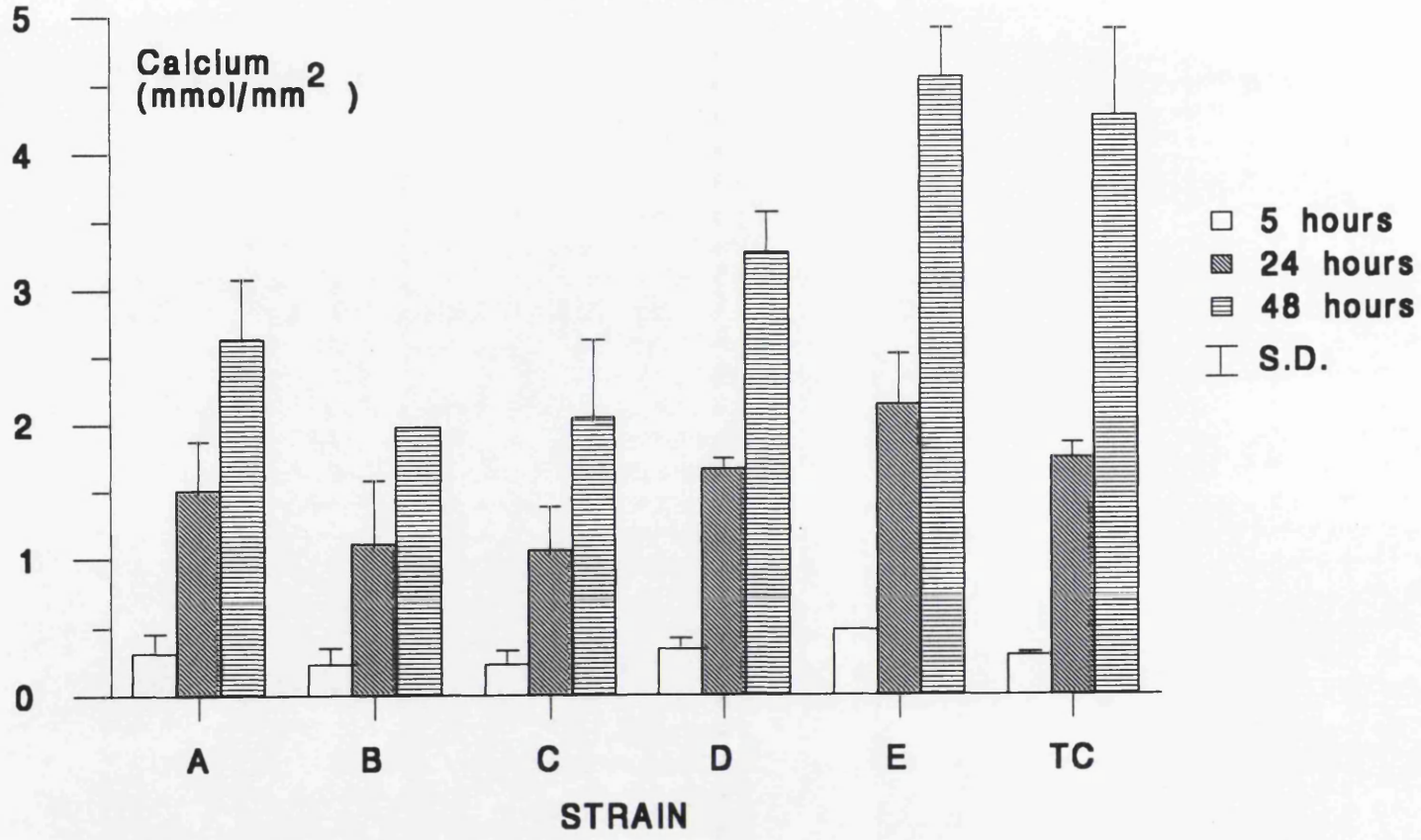


Figure 3.4. Calcium release after 5, 24 and 48 hour incubations with bovine enamel.

0.48(±0.20) mmol/mm² by strain E. Analysis of variance failed to demonstrate significant differences in the amount of calcium released, after five hours' incubation, by the six strains investigated. After the first 24 hour incubation period, mean calcium release ranged from 1.06(±0.32) mmol/mm² by strain C, to 2.14(±0.38) mmol/mm² by strain E, and by analysis of variance significant differences ($p < 0.01$) could be demonstrated in the amount of calcium released by the strains. Multiple range analysis by 95% Scheffé test showed calcium release by strain E to be significantly different from that of strains B and C.

Mean calcium release from the bovine enamel slab over the total 48 hour incubation period ranged from 1.98 mmol/mm² by strain B, to 4.56 (±0.36) mmol/mm² by strain E. By analysis of variance, a more significant discrimination between strains was achieved ($p < 0.001$). Furthermore, 95% Scheffé tests revealed strains E and TC to be significantly different from strains A, B and C.

Acid anion production during incubation with bovine enamel.

Acid production after the first five hours incubation ranged from 92(±23) nmol/mg wet weight bacteria by strain C, to 148 (±39) nmol/mg wet weight bacteria by strain E. Analysis of variance failed to demonstrate any

significant differences in acid production between strains.

Mean acid anion production during each of the 24 hour incubation periods ranged from 117 (±34) nmol/mg wet weight bacteria by strain C, to 198 (±37) nmol/mg wet weight bacteria by strain E. By Students' t-tests, differences between strains were as shown in Table 3.3. Strain E again produced the highest concentration of acids, and was significantly different from strains A, B, C and D. However, the mean acid production by strain D was not significantly different from strains A, B or C. By analysis of variance, significant differences between strains were demonstrable ($p < 0.002$). Multiple range analysis by 95% Scheffé test showed strain E to be significantly different only from strain C.

Min pH during incubation with bovine enamel.

The minimum pH recorded occurred after five hours' incubation, with the pH of the vials rising slightly by the end of the 24 hour incubation period. As detailed in Table 3.4, mean minimum pH values ranged from 3.73(±0.14) by strain E, to 4.12(±0.33) by strain B. Whilst analysis by Students' t-tests showed differences between strains E and TC, and Strain C, analysis of variance failed to demonstrate significant differences between strains ($p = 0.064$).

Strain	Acid anions (nmol/mg wet weight)	
	mean[a]	(S.D.)
A	139	(36)
B	130	(45)[b]
C	117	(34)
D	138	(17)
E	198	(37)
TC	185	(35)

Significance of differences between strains as assessed by Students' t-tests.

E v -A** -B* -C**** -D***

TC v -A* -C*** -D**

**** = p < 0.005
*** = p < 0.01
** = p < 0.02
* = p < 0.05

Significance of differences between strains as assessed by analysis of variance.

Degrees of freedom = 5, 29
F ratio = 5.15
p < 0.002

By 95% Scheffé test
Strain E significantly different from strain C.

[a] = mean of six replicates
[b] = mean and standard deviation of five replicates
S.D. = Standard Deviation
nmol/mg wet weight = nanomoles acid anions per milligram wet weight of bacteria.

Table 3.3. Acid anion production during initial and subsequent 24 hour incubations with bovine enamel.

S. mutans Strain	Min pH	
	Mean[a]	(S.D.)
A	3.94	(0.29)
B	4.12	(0.33)
C	4.03	(0.22)
D	3.89	(0.22)
E	3.73	(0.14)
TC	3.77	(0.15)

Significance of differences between strains as assessed by Students' t-tests.

E v -C**
TC v -C*

** = $p < 0.02$
* = $p < 0.04$

Significance of differences between strains as assessed by analysis of variance.

Degrees of freedom = 5, 29
F ratio = 2.37
$p = 0.064$

[a] = mean of six replicates
S.D. = Standard Deviation

Table 3.4. Minimum pH during incubation of S. mutans strains with bovine enamel slabs.

Relationship between calcium release, acid anion production and mean minimum pH during incubation with bovine enamel.

A significant correlation was obtained between all three parameters assayed in the test, as recorded in Table 3.5. After 24 hours' incubation, significant correlations were observed between, the amount of acid produced and the calcium released ($p<0.001$); the minimum pH achieved during incubation and the amount of calcium released ($p<0.001$); and the minimum pH and acids produced ($p<0.01$).

Correlation of test parameters with past caries experience.

The DMFS data of the individuals from whom the strains had been isolated originally are also tabulated in Table 3.5. Here, significant correlations between the parameters assessed in the test and past caries experience, (expressed by DMFS), are as recorded.

3.2.5 Discussion

Of the parameters assessed, the release of calcium from the bovine enamel permitted the most significant discrimination between strains. While significant differences were evident after the first 24 hours' incubation, the significance of the differences between strains was improved by considering total calcium release over the total 48 hour incubation period.

S. mutans strain	Acid anion (nmol/mg wet weight)	Calcium ($mmol/mm^2$)	Min pH	DMFS
A	139	1.32	3.94	22
B	130	1.03	4.12	19
C	117	1.03	4.03	24
D	138	1.64	3.89	28
E	198	2.28	3.73	48
TC	185	2.13	3.77	-

	Correlation coefficient (r)	
Acid anions v min pH	- 0.91	$p < 0.01$
Calcium v min pH	0.98	$p < 0.001$
Calcium v acid anions	0.95	$p < 0.001$
DMFS v min pH	- 0.91	$p < 0.02$
DMFS v acid anions	0.93	$p < 0.01$
DMFS v calcium	0.95	$p < 0.01$

number of replicates = six

Table 3.5. Relationships between DMFS, mean minimum pH, acid anion production and calcium release after 24 hours' incubation of S. mutans strains, with bovine enamel.

In her study, Macpherson (1988), analysed calcium release as per data obtained for each 24 hour incubation period. She described the strains as fitting into three groups, A B and C, of low calcium-releasing potential; D of intermediate potential, and E which gave highest calcium release.

When the results of the present study were analysed in a similar fashion, mean calcium release ranged from 1.03 (S.D. $\pm$ 0.6) mmol/mm^2 for strain C, to 2.28 (S.D. $\pm$ 0.32) for strain E. As shown in Table 3.2, strain E was significantly different from A, B, C and D, and strain D was significantly different from strains B and C. The Type Strain (TC) was significantly different from strains A, B and C. Further scrutiny of this data by analysis of variance confirmed significant differences between strains ($p < 0.001$). Multiple range analysis by 95% Scheffé tests showed strain E to be significantly different from strains A, B and C, and strain TC to be significantly different from strains B and C.

Consideration of the calcium release data in this fashion, i.e. treating each of the 24 hour incubations as distinct entities, is open to question. The bovine enamel slabs incubated during the first, third and fifth 24 hour periods were reincubated during the second, fourth and sixth 24 hour periods respectively, and the incubations were therefore not strictly comparable.

However, in agreement with the findings of Macpherson

(1988), when calcium release by all strains in the present experiment was considered, no significant differences, (as assessed by Students' t-test), were observed in the amount of calcium released during the initial 24 hour period, i.e. 1.53 (S.D. $\pm$0.46) mmol/mm^2 compared to the value obtained for the second 24 hour period, 1.64 (S.D. $\pm$0.81) mmol/mm^2.

Macpherson (1988) reported the total calcium concentration of the vial supernatant after incubation. However, it is technically difficult to ensure that exactly 4 mm^2 of enamel is exposed, therefore the results of this experiment are reported as mmol/mm^2 exposed enamel, the mean area exposed being 4.3 (S.D. $\pm$0.43) mm^2 as measured using a stereo microscope, and the values reported are approximately one quarter those reported by Macpherson.

Considering acid production, whilst after 24 hours' incubation, significant differences in acid anion production between strains were evident, in contrast to the findings of Macpherson (1988), the present study showed acid production by strain D did not differ significantly from strains A, B or C, and appropriate statistical analysis showed that strain E was significantly different only from strain C.

Macpherson (1988) had reported significant differences in

the mean minimum pH achieved by the strains during incubation. In that study, the bacterial cells had been grown in a chemically defined medium (Terleckyj et al., 1975). However, in addition to the difficulties encountered in its preparation (as discussed in Section 3.2.3), this media was unbuffered. It was decided that in the current study, cells would be grown in Todd Hewitt Broth, which contains a sodium phosphate buffer system. In this study mean initial pH values ranged from 5.57 to 5.80, in contrast to the values of 3.72 to 4.44 reported by Macpherson (1988).

Unfortunately, hopes that the higher mean initial pH would allow a more significant discrimination between strains, were not realised (Table 3.4).

As reported in Table 3.5, significant correlations were observed between the parameters assayed in the test. These were in general agreement with the findings of Macpherson (1988), correlations being observed between assayed parameters and the past caries experience of individuals from whom the strains had been isolated originally.

In the present study only five of the six clinical isolates tested originally were available for reinvestigation. The non-availability of one isolate, which was reported as being of medium / high cariogenic potential (Macpherson 1988), may have resulted in a less significant demonstration of differences between strains.

In addition, the change in range of DMFS values available meant, in effect, that the significant correlation between DMFS and test parameters was dependant heavily on strain E. Furthermore, the use of correlation coefficients to compare five strains should be interpreted with caution (Brown & Swanson Beck, 1990).

The concept of enamel dissolution *in vitro* as a means of assessing cariogenic potential, has a long history (Fosdick *et al.*, 1937, Dewar, 1949). The model described in this study is a modification of a screening test developed for the determination of the cariogenic potential of foods, (Primrose, Geddes & Weetman, 1989). The results of the present study confirm that, using this system, it is possible to investigate physiological and metabolic characteristics of *S. mutans* strains. The finding by Macpherson (1988), that parameters assessed in this test correlated significantly with past caries experience, suggested that the test could be used to investigate the cariogenic potential of microorganisms. It is proposed that such information may lead to an improvement in the sensitivity and specificity of current caries activity tests based solely on microbiological counts (see Section 1.18).

Bovine enamel is widely used in the experimental investigation of caries-related processes, and its use in the *in vitro* assessment of the cariogenic potential of

foods was recommended by the Scientific Consensus Conference on Methods for Assessment of the Cariogenic Potential of Foods (Featherstone 1986), in San Antonio, Texas, USA. It has been proposed that the use of bovine enamel in such tests could be advantageous in that it is of more uniform composition than human enamel, having been less exposed to fluoride (Mellberg & Loertscher, 1974). It also demineralises with the characteristics of a natural carious lesion, but does so three times faster (Featherstone & Mellberg, 1981). The relative scarcity of human enamel has also been cited as a reason for using bovine enamel (Brudevold et al., 1984).

Despite the above recommendations there may be, at least theoretically, differences in the demineralisation characteristics within different areas of a tooth; between different teeth, and between teeth from different animals. Hence, such variations may mask differences in the cariogenic potential of strains being tested. Patel, Fox and Higuchi (1987), have reported within-tooth and between-tooth variability of 5% and 4% respectively, for bovine enamel. However, Brudevold et al., (1984), reported that in preselecting bovine enamel blocks with a low iodide permeability for use in an intraoral demineralisation test, normally twice the number of blocks employed in a test were surveyed, indicating considerable variability. This potential variability is not mentioned by many authors. In the study of

Macpherson (1988), all enamel blocks used in an experimental "run" were obtained from a single animal. However, she commented on the fact that differences were observed between experimental "runs", and that the reproducibility of the test required further investigation.

If the test was to be developed to investigate the cariogenic potential of a large number of strains on a longitudinal basis, then it was deemed important to ensure uniformity of the mineral substrate.

In addition to variation in solubility, further disadvantages of using bovine enamel as the mineral substrate, were the time involved in the preparation of the bovine enamel slabs (Section 3.2.3), and the difficulty in obtaining this material.

In light of these factors, it was decided to investigate alternative mineral substrates for the determination of cariogenic potential. The substrates chosen as alternatives to bovine enamel, were hydroxyapatite in a solid sintered form (Section 3.3), and as a powder (Section 3.4), Figure 3.5.

3.3 The dissolution of solid hydroxyapatite in the determination of <u>in vitro</u> cariogenic potential.

3.3.1 Introduction and aims.

The potential disadvantages of bovine enamel in the determination of cariogenic potential have been discussed

Bovine Enamel	Solid Hydroxyapatite	Powdered Hydroxyapatite

Figure 3.5. Mineral substrates investigated in the determination of <u>in vitro</u> cariogenic potential.

in Section 3.2.5. The aim of this experiment was to investigate the use of solid hydroxyapatite as a substitute for bovine enamel in assay of in vitro cariogenic potential.

3.3.2 Materials and Methods

Preparation of bacterial cells for incubation with solid hydroxyapatite.

Bacterial cells were prepared for incubation with solid hydroxyapatite as described for bovine enamel, in Section 3.2.3.

Preparation of solid hydroxyapatite

Solid hydroxyapatite, incubated as described below, was kindly prepared by Dr. S. Best, Department of Materials Science, Queen Mary College, London. The raw material (in the form of powdered hydroxyapatite code N° P34), was provided by British Charcoals and MacDonalds (Greenock, Scotland). This was isostatically pressed at 1280°C for 800 minutes, and supplied in ingots measuring 34mm x 5mm x 4mm. The ingots had a density of 100%, and surface grains which were approximately 5 μm in size. These were mounted on acrylic chucks with SuperGlue (Loctite. U.K., Welwyn Garden City, England), and sectioned under water coolant by a diamond rotatory saw (Leitz Instruments Ltd, Luton, England.) at 600 rpm with an approach speed setting of "15". This resulted in slabs measuring 2mm x

5mm x 4mm, which were stored in sterile containers until required for incubation.

Incubation of bacterial cells and hydroxyapatite.

Assay of cariogenic potential using solid hydroxyapatite, was conducted as for the bovine enamel slabs described in Section 3.2.3, the enamel slabs being substituted by the solid hydroxyapatite blocks.

Statistics.

Statistical analysis was conducted as described in Section 2.4.2. The comparison of multiple groups in this, and subsequent studies in this thesis, were conducted by analysis of variance only (see Section 3.2.3).

3.3.3 Results

Calcium release during incubation with solid hydroxyapatite.

Calcium release data after 5, 24 and 48 hour incubations with the solid hydroxyapatite, are reported in Table 3.6 and Figure 3.6. Mean calcium release after the initial 5 hour incubation period ranged from 3.32 ($\pm$0.55) mmol calcium by strain C, to 5.02 ($\pm$0.76) mmol calcium by strain E. After 24 hours' incubation, calcium release ranged from 7.97 ($\pm$1.18) mmol calcium by strain C, to 10.04 ($\pm$0.48) mmol calcium by strain E. After 48 hours' incubation, minimum and maximum calcium release, again by

Calcium (mmol).						
	5 hours		24 hours		48 hours	
S. mutans Strain	mean[a]	(S.D.)	mean[a]	(S.D.)	mean[a]	(S.D.)
A	4.04	(0.71)	8.03	(1.39)	11.36	(1.17)
B	3.70	(0.53)	8.54	(0.70)	10.86	(0.53)
C	3.32	(0.55)	7.97	(1.18)	10.69	(1.14)
D	4.10	(0.90)	9.16	(0.66)	12.69	(0.83)
E	5.02	(0.76)	10.04	(0.48)	13.21	(1.00)
TC	3.43	(0.83)	9.25	(0.97)	12.32	(1.57)

Significance of differences between strains as assessed by analysis of variance.

Incubation period (hours)	5	24	48
Degrees of freedom	5, 12	5, 12	5, 12
F ratio	2.17	2.14	2.70
p value	0.126	0.130	0.074

[a] = mean of three replicates
S.D. = Standard Deviation
mmol = total calcium content in vial supernatant.

Table 3.6. Calcium release after 5, 24 and 48 hours' incubation with solid hydroxyapatite.

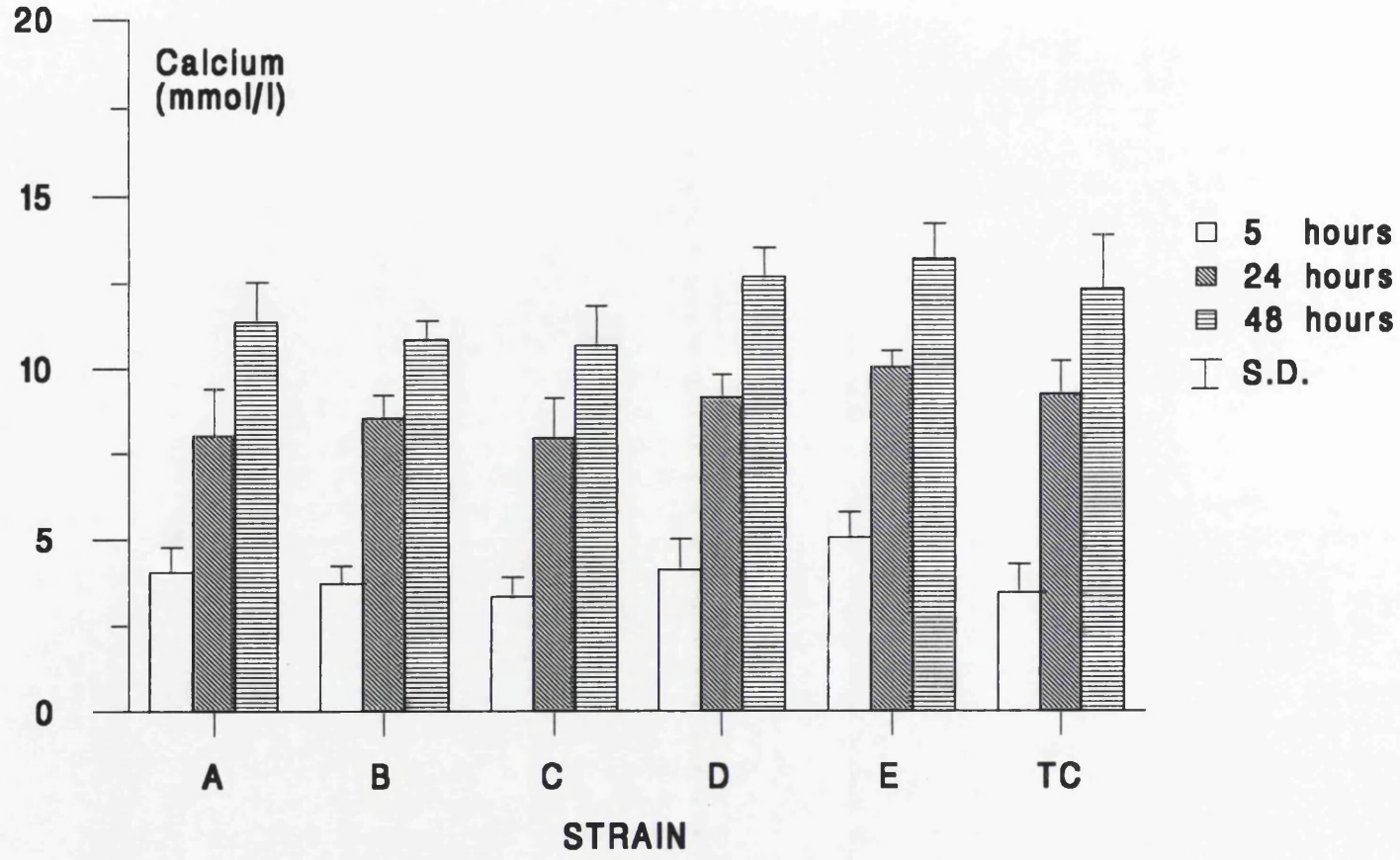


Figure 3.6. Calcium release after 5, 24 and 48 hour incubations with solid hydroxyapatite.

strains C and E respectively, ranged from 10.69 (±1.14) mmol calcium to 13.21 (±1.00) mmol calcium.

Acid production during incubation with solid hydroxyapatite.

Significant discrimination between strains, on the basis of acid production, was possible after 24 hours' incubation ($p<0.007$), values ranging from 98 (±16) nmol/mg wet weight bacteria by strain C, to 180 (±26) nmol/mg wet weight bacteria by strain E (Table 3.7).

pH during incubation with solid hydroxyapatite.

Initial pH values ranged from 5.42 to 5.86, with a mean of 5.69 (±0.15), and after 5 hours' incubation a mean minimum pH of 4.02 (±0.17) had been achieved, rising to a mean final pH of 4.08 (±0.15) after 24 hours' incubation. However, no significant differences were observable in the minimum pH achieved by the six strains under investigation.

3.3.4 Discussion.

In contrast to the findings with calcium release from the bovine enamel slabs, at none of the time intervals studied was it possible to discriminate significantly between strains on the basis of calcium release from solid hydroxyapatite. Values for calcium release, reported in Table 3.6, represent the total calcium concentration in the vial supernatant.

S. mutans Strain	Acid anions (nmol/mg wet weight).	
	mean[a]	(S.D.)
A	146	(25)
B	163	(10)
C	98	(16)
D	139	(10)
E	180	(26)
TC	144	(25)

Significance of differences between strains as assessed by analysis of variance.

Degrees of freedom = 5, 12
F ratio = 5.59
$p < 0.007$

By 95% Scheffé test

Strain E significantly different from strain C

[a] = mean of three replicates
S.D. = Standard Deviation
nmol/mg wet weight = nanomoles acid anions per milligram wet weight of bacteria.

Table 3.7. Acid production after initial 24 hour incubations with solid hydroxyapatite by S. mutans strains.

The total surface area of the hydroxyapatite block exposed to the products of bacterial metabolism was 76 mm^2, which contrasts with the 4 mm^2 of exposed bovine enamel surface in the original experiment (Section 3.2). When the mean calcium release from solid hydroxyapatite was compared with bovine enamel, e.g. following 24 hours' incubation of strain E, 12.6 times less calcium was released per square millimetre of solid hydroxyapatite than occurred from an equivalent area of bovine enamel. Hence, it was apparent that the solid hydroxyapatite was much less soluble than the bovine enamel.

When calcium release by all strains during the initial 24 hour incubation period (8.83 ($\pm$0.10) mmol calcium) was compared with calcium release during the second 24 hour incubation period (3.02 ($\pm$0.91) mmol calcium) Students' t-test revealed significant differences in the amount of calcium released ($p < 0.001$). It would appear therefore, that the susceptibility of the solid hydroxyapatite to demineralisation decreased with time. These results contrast with the bovine enamel findings described in Section 3.2.5, where no significant differences were observed in the quantity of calcium released during the initial, and second 24 hour incubation periods.

Acid production values obtained during incubation with solid hydroxyapatite were similar to those during incubation with bovine enamel. In agreement with the

bovine enamel findings, no significant differences were observable in the minimum pH achieved by the six strains under investigation.

In addition to the failure of this experiment to discriminate between the S. mutans strains on the basis of calcium release, the preparation of this form of hydroxyapatite was complex, requiring specialist equipment. Thus there is likely to be a problem of supply, should a large number of test strains require to be studied in a longitudinal assessment. It was concluded, therefore, that solid hydroxyapatite in the form tested in this experiment, was unsuitable as a substitute for bovine enamel in the determination of in vitro cariogenic potential.

3.4 The dissolution of powdered hydroxyapatite in the determination of in vitro cariogenic potential.

3.4.1 Introduction and aims.

The potential disadvantages of bovine enamel, and the unsuitability of solid hydroxyapatite in the determination of S. mutans in vitro cariogenic potential have been discussed in Sections 3.2.5 and 3.3.4. Thus the aim of this experiment, was to investigate the dissolution of powdered hydroxyapatite in the determination of in vitro cariogenic potential.

3.4.2 Materials and methods

Preparation of bacterial cells for incubation with powdered hydroxyapatite.

The preparation of bacterial cells for incubation with powdered hydroxyapatite, was essentially the same as that employed with bovine enamel, but modified as described below to produce a pellet of 40 mg wet weight (Figure 3.7).

A heavy inoculum of the strain under investigation was transferred to two Columbia blood agar plates, and incubated for 24 hours in an atmosphere of air and 5% CO_2 at 37°C. Cells were then transferred to a universal bottle which contained 20 ml Todd Hewitt Broth.

After incubation for 18 hours at 37°C in an orbital shaker (100 rpm), the universal container was centrifuged at 700*g* in an MSE Centaur centrifuge for 10 minutes. The supernatant was then discarded and cells resuspended in 10 ml 135 mM KCl (Marsh et al., 1982). The cells were then centrifuged as before. The supernatant was again discarded, and the pellet resuspended in 0.5 ml 135 mM KCl, and transferred to a pre-weighed plastic vial, which was centrifuged finally at 2000 g in an MSE HS18 centrifuge, for 20 minutes at 4°C. The supernatant was discarded and the weight of the resulting pellet adjusted to 40 mg wet weight, prior to incubation.

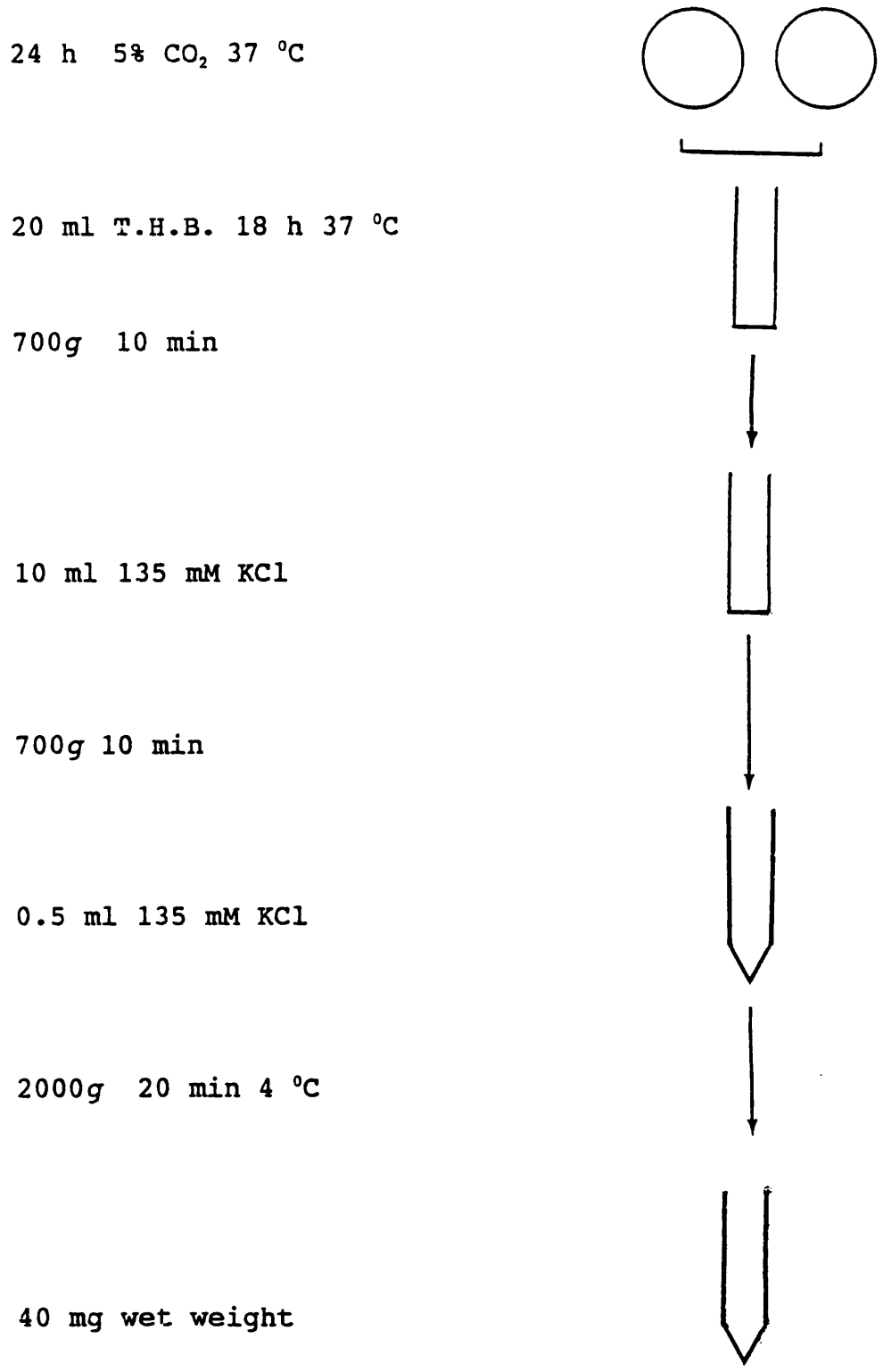


Figure 3.7. Diagrammatic representation of the preparation of bacterial cells for incubation with powdered hydroxyapatite.

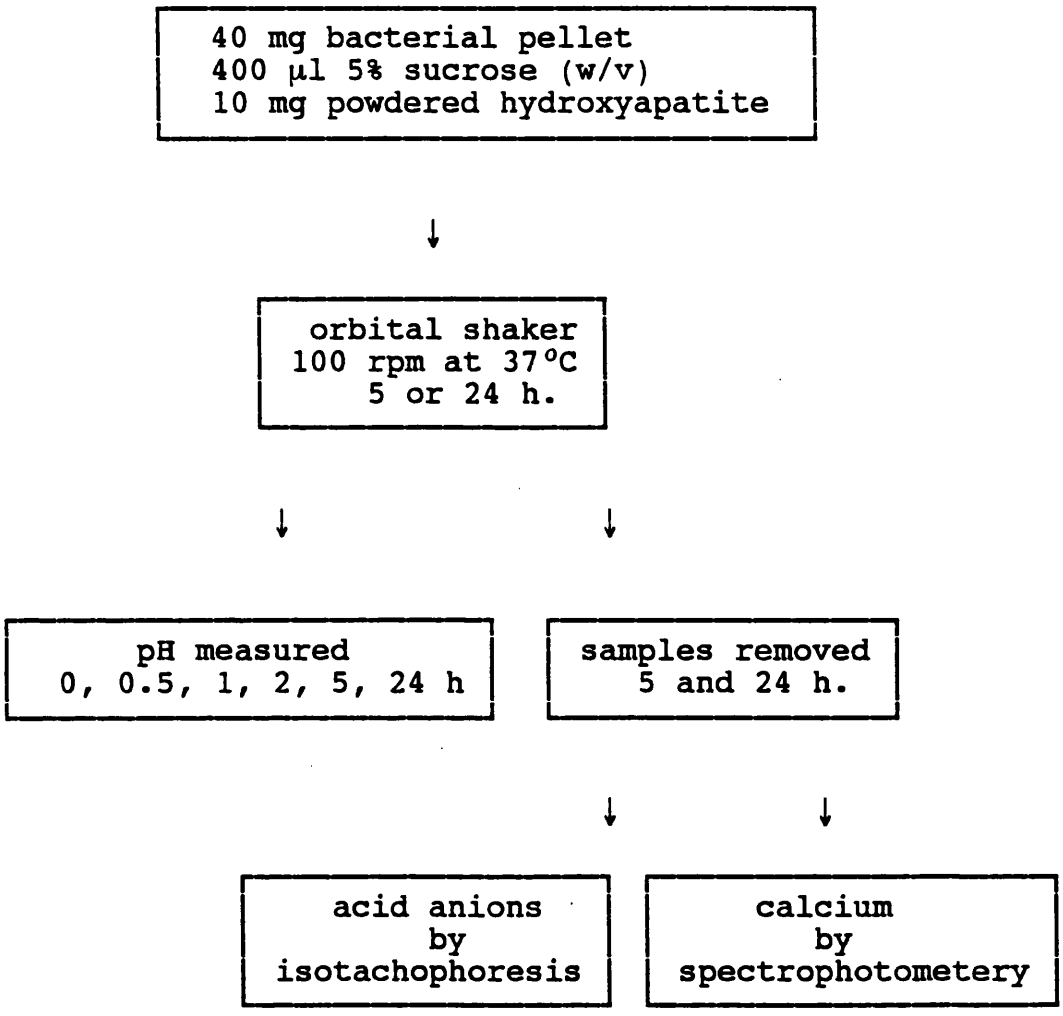


Figure 3.8. <u>In vitro</u> assessment of <u>S. mutans</u> cariogenic potential with powdered hydroxyapatite as the mineral substrate.

Preparation of powdered hydroxyapatite.

The hydroxyapatite, supplied by BDH Chemicals Ltd. England, was in the form of a fine powder. Prior to weighing, the storage container was agitated lightly to counteract any effects of settling, and 10 mg hydroxyapatite portions were weighed on an Galaxy 160 balance (OHAUS Scale Corporation, Florham Park, New Jersey), designed to measure in 0.1 mg increments. The 10 mg aliquots were stored in small sterile plastic vials (ref. N°. 72.700 Sarstedt, West Germany).

Incubation of bacterial cells and powdered hydroxyapatite.

The assay of cariogenic potential, with powdered hydroxyapatite as the mineral substrate, was conducted in a similar fashion to that described for bovine enamel in Section 3.2, but with the modifications detailed below. To the bacterial pellet, (prepared as described above), was added 400 µl of a 5% sucrose solution (w/v), and the vial agitated for 10 seconds using a Spinmix to ensure adequate mixing. Thereafter, 10 mg of powdered hydroxyapatite was added and the vial agitated again using the Spinmix. The pH was measured, and the vial incubated at 37°C for either 5 or 24 hours, in an orbital shaker (100 rpm) at 37°C. The pH was determined again after 0.5, 1, 2, 5 and 24 hours (Figure 3.8). Following incubation, the vial was stored at -30°C. Prior to determination of calcium release and acid anion

production, the vial was allowed to thaw before being centrifuged twice at 1500*g* for 20 minutes at 4°C. The supernatant, free of all debris, was then analysed for acid anion production and calcium release (see Sections 2.3 and 2.6).

3.4.3 Results

Calcium release during incubation with powdered hydroxyapatite.

Calcium release data following 5 and 24 hours' incubation with powdered hydroxyapatite are detailed in Table 3.8 and Figure 3.9. Mean calcium release after 5 hours' incubation ranged from 0.66(±0.27) mmol/mg of hydroxyapatite by strain C, to 2.01 (±0.19) mmol/mg hydroxyapatite by strain E. After 24 hours' incubation, mean calcium release ranged from 1.88 (±0.52) mmol/mg by strain B, to 3.62 (±0.72) mmol/mg by strain E. At both time intervals there were significant differences, as assessed by analysis of variance, between strains in the amount of calcium released. However, discrimination between strains was more significant after 5 hours' incubation ($p < 0.001$), when compared with 24 hours' incubation ($p < 0.01$). After 5 hours' incubation by 95% Scheffé tests strain E was shown to be significantly different from strains A, B and C.

Calcium (mmol/mg).				
	5 hours		24 hours	
S. mutans Strain	mean[a]	(S.D.)	mean[a]	(S.D.)
A	0.94	(0.31)	2.38	(0.33)
B	0.80	(0.16)	1.88	(0.52)
C	0.66	(0.27)	1.93	(0.74)
D	1.17	(0.34)	2.53	(0.62)
E	2.01	(0.19)	3.62	(0.72)
TC	1.45	(0.26)	3.44	(0.32)

Significance of differences between strains as assessed by analysis of variance.

Incubation period (hours)	5	24
Degrees of freedom	5, 12	5, 12
F ratio	10.67	5.16
p value	0.001	0.01

Significant differences by 95 % Scheffé tests.

After 5 hours E v A, B and C

[a] = mean of three replicates
S.D.= Standard Deviation
mmol/mg = millimoles per milligram of hydroxyapatite

Table 3.8. Calcium release after 5 and 24 hours' incubation with powdered hydroxyapatite by S. mutans strains.

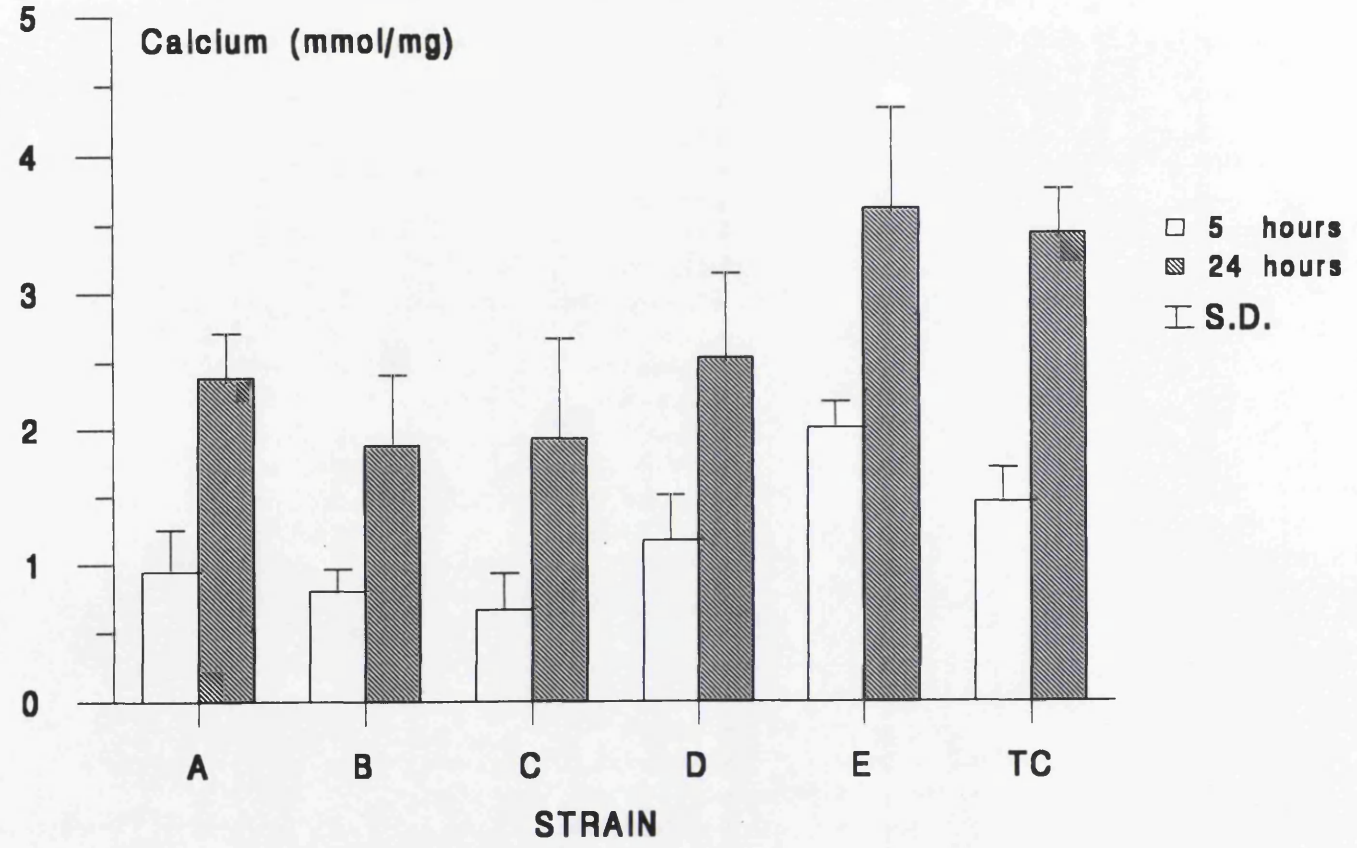


Figure 3.9. Calcium release after 5 and 24 hours' incubation with powdered hydroxyapatite.

Acid production during incubation with powdered hydroxyapatite.

Acid production after 5 and 24 hours' incubation by strains A-TC, is reported in Table 3.9. Mean acid anion production after 5 hours' incubation ranged from 229 (±65) nmol/mg wet weight bacteria by strain C, to 520 (±35) nmol/mg wet weight by strain E. After 24 hours' incubation values ranged from 756 (±396) nmol/mg wet weight bacteria by strain C, to 1354 (±259)nmol/mg wet weight bacteria by strain E. Significant differences in acid production by the six strains under investigation were demonstrable after 5 hours' incubation. By 95% Scheffé tests strain E was shown to be significantly different from strains B and C. After 24 hours' incubation, analysis of variance failed to demonstrate significant differences in acid production between strains.

pH during incubation with powdered hydroxyapatite.

The pH of the vials after 5 and 24 hours' incubation were as listed in Table 3.10. After 5 hours' incubation, pH ranged from 4.45 (±0.17) for strain C, to 4.02 (±0.03) for strain E. After 24 hours' incubation, pH values ranged from 4.08 (±0.15) to 3.78 (±0.14), again by strains C and E respectively. By analysis of variance, it was possible to demonstrate significant differences between strains after 5 hours' incubation and 95% Scheffé tests showed strain E to be significantly

Acid anions (nmol/mg wet weight).				
	5 hours		24 hours	
S. mutans Strain	mean[a]	(S.D.)	mean[a]	(S.D.)
A	331	(87)	974	(242)
B	267	(56)	806	(383)
C	229	(65)	756	(396)
D	378	(96)	894	(367)
E	520	(35)	1354	(259)
TC	405	(64)	1138	(146)

Significance of differences between strains as assessed by analysis of variance.

Incubation period (hours)	5	24
Degrees of freedom	5, 12	5, 12
F ratio	6.64	1.55
p value	0.003	0.25

Significant differences by 95 % Scheffé tests.

After 5 hours E v B and C

[a] = mean of three replicates
S.D. = Standard Deviation
nmol/mg wet weight = nanomoles acid anions per milligram wet weight bacteria

Table 3.9. Acid production following 5 and 24 hours' incubation with powdered hydroxyapatite by S. mutans strains.

	pH			
S. mutans	5 hours		24 hours	
Strain	mean[a]	(S.D.)	mean[a]	(S.D.)
A	4.23	(0.05)	3.83	(0.19)
B	4.38	(0.05)	4.03	(0.18)
C	4.45	(0.17)	4.08	(0.15)
D	4.24	(0.06)	3.99	(0.16)
E	4.02	(0.03)	3.78	(0.14)
TC	4.15	(0.02)	3.84	(0.12)

Significance of differences between strains as assessed by analysis of variance.

Incubation period (hours)	5	24
Degrees of freedom	5, 12	5, 12
F ratio	11.03	1.83
p value	0.001	0.18

Significant differences by 95 % Scheffé tests.

After 5 hours	E v B and C TC v C

[a] = mean of three replicates
S.D.= Standard Deviation

Table 3.10. pH after 5 and 24 hours' incubation with powdered hydroxyapatite by S. mutans strains.

different from strains B and C. Strain TC data were also significantly different from those of strain C. However, by 24 hours, differences in pH between strains were no longer significant.

Correlation of test parameters with past caries experience.

As reported in Table 3.11, a significant correlation between parameters assessed in the test and past caries experience, could again be demonstrated. However, the caution required in interpreting that data as was discussed in Section 3.2.5, is equally valid.

3.4.4 Discussion.

During the assay of cariogenic potential with bovine enamel, 90 mg wet weight of bacterial cells were incubated with 270 µl of 5% w/v sucrose solution, as this has been reported to result in an acid concentration similar to that found in dental plaque *in vivo*, (Primrose *et al.*, 1989). In this experiment, the preparation of bacterial cells for incubation with powdered hydroxyapatite was modified, as described in Section 3.4.2. The ratio of wet weight of bacterial pellet : weight of hydroxyapatite : volume of the sucrose solution chosen was, in this experiment, determined by practical considerations aimed at simplifying the original test. However, this modified protocol meant that direct comparison of acid production and pH changes with those

Strain	Acid anion (nmol/mg wet weight)	Calcium (mmol/mg)	Min pH	DMFS
A	331	0.94	4.23	22
B	267	0.80	4.38	19
C	229	0.66	4.45	24
D	378	1.17	4.24	28
E	520	2.01	4.02	48
TC	405	1.45	4.15	-

	Correlation coefficient (r)	
Acid anions v min pH	- 0.95	$p < 0.01$
Calcium v min pH	0.98	$p < 0.001$
Calcium v acid anions	0.98	$p < 0.001$
DMFS v min pH	- 0.95	$p < 0.01$
DMFS v acid anions	0.85	$p < 0.05$
DMFS v calcium	0.91	$p < 0.01$

number of replicates = three

Table 3.11. Relationships between mean pH, acid anion production and calcium release after 5 hours' incubation with powdered hydroxyapatite and DMFS.

observed during bovine enamel assays were not possible. It is interesting that Reynolds and Riley (1981) have shown that the rate of acid production by a strain of S. mutans incubated with 500 mg hydroxyapatite was saturated at a sucrose concentration of 2 mM which was, of course, exceeded by the sucrose concentration used in this experiment. While alteration of the chosen ratio of bacteria, hydroxyapatite and carbohydrate, (and of carbohydrate concentrations), may have resulted in a more significant discrimination between strains, constraints of time precluded investigation of the numerous permutations possible.

As outlined in Table 3.12, the results of the experiment using powdered hydroxyapatite showed that it was possible to discriminate significantly between strains on the basis of calcium release after only 5 hours' incubation with powdered hydroxyapatite ($p < 0.001$), and whilst significant differences between strains were still apparent after 24 hours, the level was reduced ($p < 0.01$). Significant differences in acid production ($p < 0.003$), and pH ($p < 0.001$) were demonstrable after 5 hours' incubation, whereas by 24 hours, the differences between strains were no longer significant. This again contrasts with acid production in the bovine enamel assay, where it was only possible to discriminate significantly between strains on the basis of acid production after 24 hours' incubation.

Calcium release (p value)			
Mineral	5 hours	24 hours	48 hours
Bovine Enamel	NS	0.01	0.001
Solid Hydroxyapatite	NS	NS	NS
Powdered Hydroxyapatite	0.001	0.01	-

Acid anions (p value)		
Mineral	5 hours	24 hours
Bovine Enamel	NS	0.01
Solid Hydroxyapatite	NS	0.007
Powdered Hydroxyapatite	0.003	NS

pH (p value)		
Mineral	5 hours	24 hours
Bovine Enamel	NS	NS
Solid Hydroxyapatite	NS	NS
Powdered Hydroxyapatite	0.001	NS

NS = not significant.

Table 3.12. Discrimination between S. mutans strains on the basis of calcium release, acid anion production and pH during incubations with mineral substrates, as assessed by analysis of variance.

It is apparent therefore, that in the case of powdered hydroxyapatite, the significance of differences between strains decreased with time, in contrast to the findings with bovine enamel, where the significance of differences in calcium release and acid production increased with time (Table 3.12).

A comparison of results presented from this study, with those obtained using the bovine enamel is complicated further by the findings of Berry and Henry (1977), who demonstrated enhanced metabolic activity of S. mutans strains adsorbed to hydroxyapatite, as compared to control suspensions without hydroxyapatite. Furthermore, the impossibility of determining accurately, the surface area of the powdered hydroxyapatite, prevented a direct comparison of calcium release on the basis of unit area. However, the total calcium content after 5 hours' incubation of the vial containing strain E was higher, (by a factor of approximately times ten), with the powdered hydroxyapatite, than with the bovine enamel.

By substituting powdered hydroxyapatite for bovine enamel, the ability to create a lesion which mimicked a natural caries lesion was lost. However, since the test aimed to assess factors that relate to the cariogenic potential of the strains, rather than the ability to create a true caries lesion, this loss is probably not of importance. Macpherson (1988), had assessed mineral loss

from bovine enamel slabs by microradiography, but reported that the variation encountered prohibited the use of this technique to identify differences in the demineralising potential of the S. mutans strains investigated. From visual inspection of the slabs employed here, following incubation the exposed enamel window had an etched appearance, and it appeared that surface erosion occurred during the test, rather than the formation of a subsurface lesion.

3.5 Conclusions

The significance of the differences between the six strains of S. mutans investigated in this chapter, as per calcium release (Figures 3.4, 3.6 & 3.9); acid anion production, or pH changes when incubated with bovine enamel, solid hydroxyapatite or powdered hydroxyapatite, are summarised in Table 3.12. From the foregoing discussions, it is apparent that after only 5 hours incubation with powdered hydroxyapatite it is possible to discriminate significantly between strains on the basis of all three parameters investigated.

As outlined in Section 1.19 the aim of this thesis was to investigate the in vitro cariogenic potential of a large number of strains of S. mutans. In light of the findings in these experiments and, in addition to the ease of obtaining and preparing the powdered hydroxyapatite, it was decided that future in vitro cariogenic potential

investigations would be undertaken employing powdered hydroxyapatite, incubated for a period of 5 hours.

This model was then used to assay the *in vitro* cariogenic potential of six *Streptococcus* species (Chapter 4); of dental plaque collected from 12 year-olds (Chapter 5), and of strains of *S. mutans* isolated from children with a range of caries experience (Chapters 6 and 7).

CHAPTER 4

THE *IN VITRO* CARIOGENIC POTENTIAL OF *STREPTOCOCCUS* SPECIES.

4.1 An investigation of the *in vitro* cariogenic potential of *Streptococcus* species.

4.1.1 Introduction

Many studies have investigated the role of the mutans streptococci in the caries process and a wealth of information on these species has accumulated. However whilst their importance in the disease is without doubt, it has been shown that other oral streptococcal species can produce caries in gnotobiotic rats (Willcox, Drucker & Green, 1990). Streptococci usually comprise a substantial proportion of the viable cell count in dental plaque (Hardie *et al.*, 1977; Mikkelsen, Jensen & Jakobsen, 1981; Macpherson *et al.*, 1991), and recent findings of early enamel demineralisation in the absence of detectable levels of *S. mutans*, (Marsh *et al.*, 1989; Macpherson *et al.*, 1990), again raise the question of the contribution of streptococcal species, other than mutans streptococci, to the caries process.

The taxonomy of the viridans streptococci has recently undergone much reclassification, as advances in molecular biological techniques have provided the ability to classify bacteria on the basis of genetic similarity, in contrast to traditional classification on phenotypic

grounds (Coykendall, 1989). This Chapter describes the investigation of the cariogenic potential of six streptococcal species (listed in Table 4.1), using the *in vitro* test system described in Section 3.4.

4.1.2 Materials and methods

The six streptococcal strains used in this investigation were obtained as freeze-dried cultures from the National Collection of Type Cultures (London, England).

Strains were prepared for determination of *in vitro* cariogenic potential as described in Section 3.4., except that Todd Hewitt Broth manufactured by Oxoid (Basingstoke, England), Lot Number 116 41635, was used instead of that supplied by Gibco.

Strains were incubated in six experimental batches designated (1-6). Each strain was investigated on six separate occasions and was assigned to the batches as in Table 4.2, to minimise potential variation due to batch effect.

Statistical analysis was conducted as described in Section 2.4.2.

4.1.3 Results

The cariogenic potential of the species was assessed on the basis of acid production, calcium release and pH after 5 hours' incubation with powdered hydroxyapatite. The relationship between mean acid production, calcium release and pH by each of the species investigated, is

*E. faecalis	NCTC	775
S. gordonii	NCTC	3165[a]
S. sanquis	NCTC	7863
S. vestibularis	NCTC	12166
S. mutans	NCTC	10449
S. sobrinus	NCTC	10922

[a] strain NCTC 3165 was originally designated as the type strain of S. mitis and has recently been redesignated as S. gordonii. (Kilian et al 1989)

*see page 23

Table 4.1. Strains of Streptococcus species investigated in Sections 4.1 and 4.2.

Experimental Batch	Species					
1	1	2	3	4	5	6
2	1	1	2	2	3	3
3	4	4	5	5	6	6
4	1	2	3	4	5	6
5	1	1	2	2	3	3
6	4	4	5	5	6	6

1 = S. faecalis
2 = S. gordonii
3 = S. mutans
4 = S. sanquis
5 = S. sobrinus
6 = S. vestibularis

Table 4.2. Design of experiment described in Section 4.1.

illustrated in Figure 4.1. The correlation coefficients and significance of the relationships are recorded in Table 4.3.

Calcium release by each of the six species, recorded as the mean calcium concentration of the vial following incubation, is reported in Table 4.4 and ranged from 3.10 (±1.18) mmol/l by E. faecalis, to 41.52 (±4.57) mmol/l by S. mutans. By analysis of variance, species could be arranged in three homogeneous groups, S. faecalis, S.sanguis, and S. gordonii of lowest cariogenic potential, S. vestibularis of intermediate cariogenic potential, and S. mutans and S. sobrinus of highest cariogenic potential. In Table 4.5, calcium release data are presented as calcium release per milligram wet weight of bacteria incubated. Whilst a higher mean calcium release by the S. sobrinus strain than the S. mutans strain resulted, no difference in the significance of the values ensued from presenting the data in this fashion.

Mean acid anion production, expressed as total acid anions, is reported in Table 4.6 and ranged from 226 (±77) nmol/mg wet weight bacteria by S. faecalis, to 1257 (±80) nmol/mg wet weight bacteria by S. sobrinus. By analysis of variance, it was again possible to assign the species to three homogeneous groups. However, the variability in acid production by S. vestibularis meant, that in contrast to the calcium results, this species

* see page 23

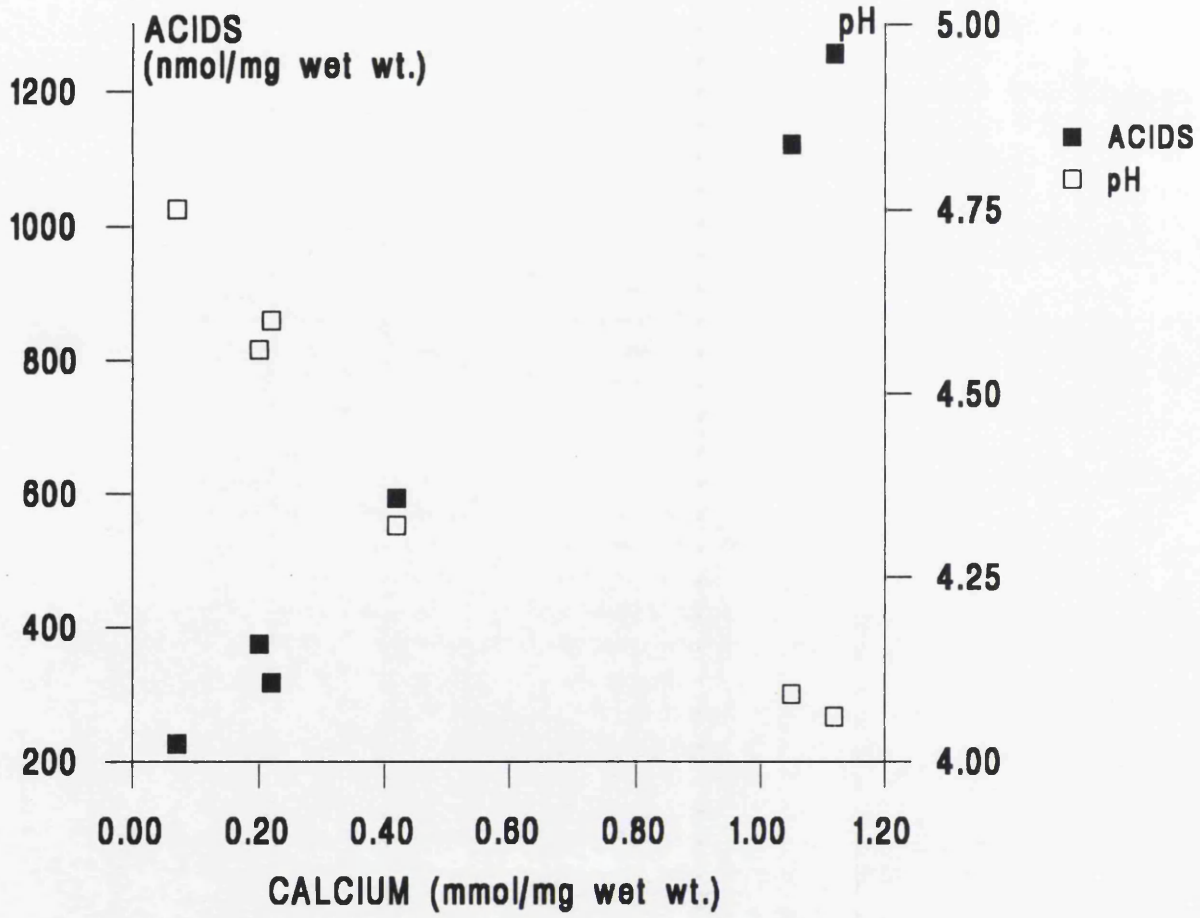


Figure 4.1. Relationship of acid production, calcium release and pH after 5 hours' incubation of six species of streptococci.

	Correlation coefficient	p value
Calcium vs acids	0.996	0.001
Calcium vs pH	-0.965	0.001
Acids vs pH	-0.972	0.001

Table 4.3. Correlation of acid anion production, calcium release and pH after 5 hours' incubation of six species of streptococci.

	Calcium (mmol/L)	
Species	mean[1]	S.D.
S. faecalis	3.10	1.18
S. sanquis	7.92	1.25
S. gordonii	7.68	1.93
S. vestibularis	15.86	4.16
S. mutans	41.52	4.57
S. sobrinus	39.30	4.00

Significance of differences between strains as assessed by analysis of variance.

Degrees of freedom	5, 30
F ratio	169
p value	0.0001

Significant differences by 95% Scheffé tests.

S. faecalis[a]

S. sanquis[a]

S. gordonii[a]

S. vestibularis[b]

S. mutans[c]

S. sobrinus[c]

Superscripts indicate homogeneous groups

[1] = mean of six replicates
S.D. = Standard Deviation

Table 4.4. Calcium release (mmol/l) by Streptococcus species after 5 hours incubation.

Calcium (mmol/mg wet weight bacteria)		
Species	mean[1]	S.D.
S. faecalis	0.07	0.02
S. sanquis	0.20	0.03
S. gordonii	0.22	0.06
S. vestibularis	0.42	0.12
S. mutans	1.05	0.12
S. sobrinus	1.12	0.13

Significance of differences between strains as assessed by analysis of variance.

Degrees of freedom	5, 30
F ratio	139
p value	0.0001

Significant differences by 95% Scheffé tests.

S. faecalis[a]

S. sanquis[a]

S. gordonii[a]

S. vestibularis[b]

S. mutans[c]

S. sobrinus[c]

Superscripts indicate homogeneous groups

[1] = mean of six replicates
S.D. = Standard Deviation

Table 4.5. Calcium release (mmol/mg wet weight bacteria) by Streptococcus species after 5 hours incubation.

Acids (nmol/mg wet weight bacteria)		
Strain	mean[1]	S.D.
S. faecalis	226	77
S. sanquis	375	123
S. gordonii	316	99
S. vestibularis	593	216
S. mutans	1121	172
S. sobrinus	1257	80

Significance of differences between strains as assessed by analysis of variance.

Degrees of freedom	5, 30
F ratio	61
p value	0.0001

Significant differences by 95% Scheffé tests.

S. faecalis[a]

S. sanquis[ab]

S. gordonii[ab]

S. vestibularis[b]

S. mutans[c]

S. sobrinus[c]

Superscripts indicate homogeneous groups

[1] = mean of six replicates
S.D. = Standard Deviation

Table 4.6. Acid production by species after 5 hours' incubation.

could not be significantly differentiated from S. sanguis or S. gordonii.

A qualitative analysis of acid anion production showed that for all species, with the exception of S. faecalis, the major acid end product was lactate, which comprised over 90% of acid anions present (Table 4.7). The second most common acid anion was acetate which accounted for 22% of acid production by S. faecalis, but less than 7% in the other species. By analysis of variance, it was possible to demonstrate that the proportion of lactate and acetate produced by the S. faecalis strain was significantly different from the other species investigated. The remainder of acid production consisted of minor quantities of formate, pyruvate, succinate and propionate.
The role of different acid anions and their contribution to the cariogenic potential of a species is discussed further in Section 6.3.4.

The pH of the vials following 5 hours' incubation is reported in Table 4.8, and ranged from 4.06(±0.10) by S. sobrinus to 4.75(±0.13) by S. faecalis. By analysis of variance, it was again possible to significantly discriminate between strains. Multiple range analysis by 95% Scheffé tests identified three homogeneous groups, which corresponded to the groups identified on the basis of calcium release, with the exception of

	Lactate mean (S.D.)		Acetate mean (S.D.)	
S. faecalis	65.1	(9.0)	22.0	(5.8)
S. gordonii	93.4	(3.7)	4.7	(2.9)
S. sanguis	93.2	(6.0)	6.6	(6.0)
S. vestibularis	95.1	(2.5)	4.1	(1.6)
S. mutans	94.9	(2.2)	2.1	(1.7)
S. sobrinus	96.7	(0.6)	1.0	(0.4)

Table 4.7. Proportion of lactate and acetate anions comprising total acid production by Streptococcus species after 5 hours' incubation.

	pH	
Species	mean[1]	S.D.
S. faecalis	4.75	0.13
S. sanquis	4.56	0.07
S. qordonii	4.60	0.11
S. vestibularis	4.32	0.15
S. mutans	4.09	0.08
S. sobrinus	4.06	0.10

Significance of differences between strains as assessed by analysis of variance.

Degrees of freedom	5, 30
F ratio	40
p value	0.0001

Significant differences by 95% Scheffé tests.

S. faecalis[a]

S. sanquis[a]

S. qordonii[a]

S. vestibularis[b]

S. mutans[bc]

S. sobrinus[c]

Superscripts indicate homogeneous groups

[1] = mean of six replicates
S.D. = Standard Deviation

Table 4.8. pH after 5 hours incubation of *Streptococcus* species.

S. vestibularis, which could not be significantly differentiated from S. mutans on the minimum pH results. The changes in mean pH during the incubation are depicted in Figure 4.2., from which it can be observed that for all species pH fell most rapidly during the first 30 minutes of the incubation. The change in pH after 30 minutes, as a percentage of the total pH fall during the 5 hours incubation, ranged from 56.7% by S. faecalis to 96.6% by S. sanquis. Whilst no significant differences in the initial pH of the vials could be detected, significant differences between species, in both the pH of the vials, and in the change in pH after 0.5, 1, 2 and 5 hours' incubation, could be demonstrated.

4.1.4 Discussion

The species studied were a combination of Streptococcus species whose cariogenic potential is well established, and some whose cariogenic potential has been less extensively investigated (Newbrun, 1989), these being listed in Table 4.1.

S. faecalis has occasionally been isolated from tooth surface fissures, although it is not thought to be a normal inhabitant of occlusal fissures (Meiers, Wirthlin & Shklair, 1982). Little information on the cariogenic potential of this species is available although Drucker and Green (1981), in an animal model reported a strain of S. faecalis as having low cariogenicity in a rat model.

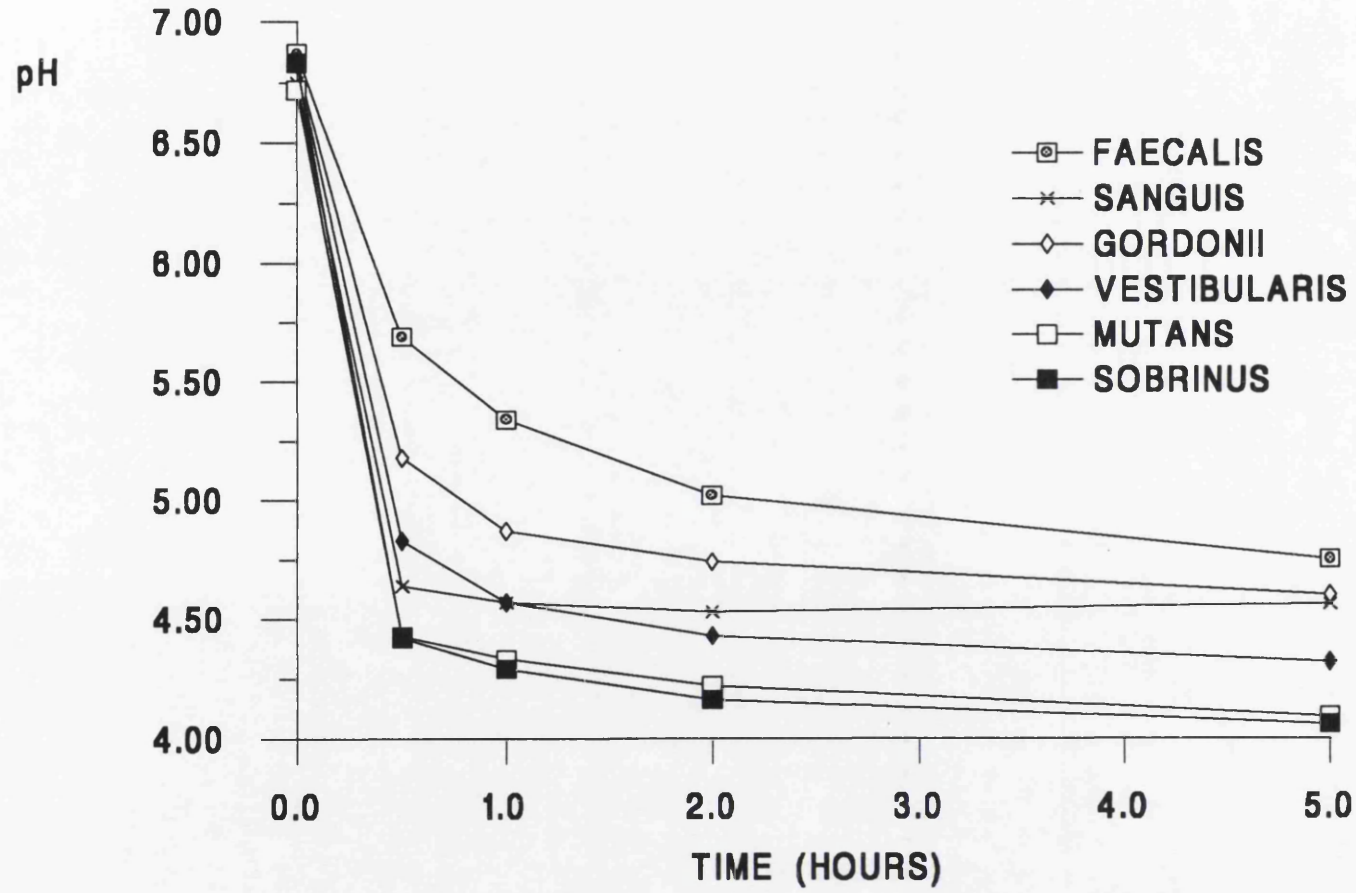


Figure 4.2. Changes in pH during incubation of six species of streptococci.

The strain NCTC 3165, was included in this study as the Type strain of S. mitis. This strain has however been shown to be atypical of that species and is actually a strain of S. sanquis (Coykendall, 1989). The latter has itself been shown to consist of two moderately related genetic groups and Kilian, Mikkelsen and Henrichsen (1989), have proposed that the group containing NCTC 3165 become a separate species named S. gordonii. Little evidence on the cariogenic potential of S. gordonii is available.

S. sanquis is one of the predominant groups of streptococci colonising the surfaces of teeth (Newbrun, 1989). Like S. mutans it produces extracelluar glucans from sucrose. Early studies on the cariogenic potential of S. sanquis were hampered by the difficulties in successfully implanting this species in a hamster model (Krasse & Carlsson, 1970). However Drucker, Shakespeare and Green (1984), found low caries scores with S. sanquis in a rat model. According to Coykendall (1989), there is no evidence that S. sanquis contributes to caries, its presence being inversely related to caries experience (Edwardsson, 1986).

S. vestibularis is a recently described species (Whiley & Hardie, 1988), the clinical importance of which awaits determination (Coykendall, 1989). The species is related to S. salivarius which, in animal models, has produced

moderate levels of caries (Drucker & Green, 1981). In the current study, calcium release by S. vestibularis, indicated this strain had an intermediate cariogenicity compared with other species tested. However, Willcox et al (1991), have reported that in gnotobiotic rats, the strain investigated in this study, (S. vestibularis NCTC 12166), produced low levels of caries.

The role of the mutans streptococci in caries has been discussed in detail in Section 1.7.

The finding that the species could be ranked into three homogeneous groups on the basis of calcium release with S. faecalis, S. gordonii and S. sanguis of low cariogenic potential, S. vestibularis of intermediate potential and S. mutans, and S. sobrinus of highest cariogenic potential, is in broad agreement with previous assessment of their cariogenicity. Drucker and Green (1981), determined the relative cariogenicity of different streptococci in a gnotobiotic rat model and concluded that, of the species tested, S. mutans was the most cariogenic and S. faecalis the least cariogenic.

As discussed in Chapter 1, recently much interest has focused on differences in the cariogenic potential of different species of the mutans streptococci. Willcox, Drucker and Green (1989), demonstrated differences not only in the cariogenic potential of different species of

mutans streptococci, but also between strains of a given species. Furthermore, they showed that S. mutans NCTC 10449 (tested in this study) was highly cariogenic. De Soet, Toors and de Graaf (1989), in an in vitro investigation of acidogenesis by oral streptococci at different pH values, concluded that a group of S. sobrinus strains, [including S. sobrinus OMZ 176 (NCTC 10922)], was more acidogenic than S. mutans, and more recently that fresh isolates of S. sobrinus are more cariogenic in rats than fresh isolates of S. mutans (De Soet et al., 1991). In contrast, Kohler and Krasse (1990) concluded that hamsters infected with fresh isolates of S. sobrinus, generally showed lower caries scores than those infected with fresh isolates of S. mutans, and that amongst the groups infected with different strains of S. mutans, the caries scores varied. However in that study (Kohler & Krasse 1990), considerable variation was observed in caries scores both between and within species and strains, and cariogenic potential was assessed on a maximum of three occasions with a number of the strains being tested only twice.
In the current exercise, it was not possible to differentiate significantly between the S. mutans and S. sobrinus strains.

Whilst the aim of the experiment was to incubate 40 mg wet weight of the organisms, this was not always technically possible, the mean weight incubated being

38.2 mg. Analysis of variance confirmed however, there were no significant differences between species with reference to the mean weight of bacteria incubated. Calcium release data have been presented as per concentration of the vial supernatant (Table 4.4), and as per the weight of organisms incubated (Table 4.5). Analysis of the data in this manner did not affect the significance of the results, the species still being allocated to the same three homogeneous groups.

Of central importance in assessing the metabolic characteristics of bacteria, is standardisation of cell cultures. This fact often receives little attention or comment in the literature and phrases such as, "cells were resuspended in the solution to give approximately 30 mg dry weight" are common. To prepare a standardised and reproducible collection of bacterial cells for study, a number of factors require consideration. The metabolism of bacteria is influenced by rate of growth (Ellwood, Phipps & Hamilton, 1979) and cells may be prepared, either by continuous culture techniques using a chemostat, or in batch culture. Continuous culture offers the major advantage that once the system has reached equilibrium, the cell number and nutrient status remain constant (Hammond, 1990). However, if large numbers of strains are to be investigated, then the establishment of a chemostat for each strain is impractical. Cells therefore have to be prepared using

batch culture techniques and, in the current experiment, the bacterial pellet was prepared using stationary phase cells. During stationary phase, bacterial cell numbers remain relatively constant and, although the cells have ceased to multiply, they retain their ability to carry on metabolic processes (Hammond, 1990).

Having produced cells in the same stage of their growth cycle, it is then necessary to prepare a standardised inoculum. It is possible, after appropriate dilution, inoculation and incubation, to obtain viable cell counts. However, the results of such a procedure are available only 2-3 days later (unless a counting chamber is used which is time consuming for routine purposes). There are three methods commonly employed to standardise inocula which permit immediate measurement and standardisation, namely optical density, wet weight and dry weight. All suffer the disadvantage that they do not account for the contribution to optical density or weight, of either extracelluar material, or non-viable cells.
In this experiment, cells were standardised on the basis of weight. However it is possible that whilst no significant differences existed in the weight of the different species incubated, that numbers of viable cells incubated differed between species. This possibility is investigated further in Section 4.2.

Changes in pH during the experiment has principally been

analysed on the basis of the vial contents pH after 5 hours' incubation. Data are presented as the arithmetic mean of the pH, as little variation was observed on repeat pH recordings (Rosen, 1963). However, as can be observed from Figure 4.2, the pH fell most rapidly during the initial incubation period and it was possible to discriminate significantly ($p < 0.001$) between the species after 30 minutes incubation. Whilst the cariogenic potential of the species as assessed by pH after 30 minutes was similar to that observed after 5 hours, the initial very rapid fall in pH observed with S. sanguis meant that, at this time its pH was lower than that observed with S. vestibularis.

Previous studies of pH fall have been reported by Marsh et al 1984, who reported pH after only 15 minutes incubation, whereas De Soet et al., 1991, measured pH after 24 hours incubation in glucose-supplemented Todd Hewitt Broth. In the current experiment, initial pH fall was very rapid, and it may be argued that the study of pH changes in this early stage of incubation may be more appropriate in determining differences in cariogenic potential between species. However incubation for such a short period may prevent the contribution of the aciduric properties of the organisms to the assessment of cariogenic potential being expressed and detected. Additionally, pH changes are only one component of this model and the relationship between pH changes, acid

production and calcium release is studied further in Section 8.1.

An alternative approach to the analysis of serial measurements was described by Matthews et al., (1990), who suggested that the use of summary data was more appropriate than comparing data at individual time points.

Using the following formula,

$$AUC = \tfrac{1}{2}\Sigma_{i=0}^{n-1}(t_{i+1}-t_i)(y_i+y_{i+1}).$$

where AUC = Area under curve

n+1 = number of measurements y_i at times t_i (i=0, ..., n).

the area under each of the pH fall curves was calculated and the data are reported as the mean area in Table 4.9. Such an approach assumes linear pH changes between successive time points. Analysis of the data in this fashion did not manifestly affect the assessment of cariogenic potential when compared with that calculated on the basis of pH after 5 hours incubation.

In the current experiment, strains were selected to represent the species investigated. Previous reports (Drucker, Shakespeare & Green, 1984; Kohler & Krasse, 1990) have indicated considerable variation in cariogenicity between strains of a given species, and it is therefore not possible to ascribe a definitive cariogenic potential to a species on the results of an

	area under pH curve (pH.hours)	
Species	mean[1]	S.D.
S. faecalis	25.7	(0.2)
S. sanquis	23.3	(0.4)
S. gordonii	24.3	(0.6)
S. vestibularis	22.9	(1.0)
S. mutans	21.7	(0.4)
S. sobrinus	21.5	(0.5)

[1] = mean of six pH fall curves
S.D. = Standard Deviation

Table 4.9. Mean area under pH curves following incubation of six species of streptococci.

individual strain of the species. In addition as discussed in Section 1.5, many factors contribute to the cariogenic potential of bacterial species. Whilst useful information may be obtained from studies such as that just described, in vivo, bacteria exist in a complex ecosystem, and the interaction of any organism with its environment will significantly influence its cariogenic potential (Marsh, Keevil & Ellwood, 1984).

4.2 Effect of nutrient media on in vitro cariogenic potential of Streptococcus species.

4.2.1 Introduction

When acid production by S. mutans NCTC 10449, described in Section 4.1 (1121 ± 172 nmol/mg wet weight bacteria), was compared with that observed in the experiment reported in Section 3.4 (405 ± 64 nmol/mg wet weight bacteria), considerable differences in total acid anion production were noted. The most obvious difference between these studies was the use of Todd Hewitt Broth supplied by two different manufacturers. With the exception of the studies described in this Chapter, and that described in Section 8.1, where the cells were grown in Todd Hewitt Broth supplied by Oxoid (Basingstoke, England), all other experiments in this thesis were conducted using Todd Hewitt Broth supplied by Gibco (Paisley, Scotland).

Kaufman et al., 1984, showed that artificial caries induction by oral streptococci in extracted human teeth was significantly influenced by the media in which the bacteria had been grown. The following study was designed to investigate the effect of growing the strains in Todd Hewitt Broth prepared by different manufacturers, on subsequent cariogenic potential.

4.2.2 Materials and methods.

The strains used in Section 4.1 (Table 4.1) were investigated on three separate occasions and grown in either Todd Hewitt Broth (Oxoid, Basingstoke, England), or Todd Hewitt Broth (Gibco, Paisley, Scotland). Bacterial cells were prepared as described in Section 3.4, prior to incubation for 5 hours in the presence of the hydroxyapatite and 5% (w/v) sucrose solution. The effect of the different brands of nutrient broth on acid anion production, calcium release and pH were then assessed.

4.2.3 Results

Acid production, calcium release and pH change after 5 hours incubation of streptococcal species previously cultured in either Todd Hewitt Broth (Oxoid) or Todd Hewitt Broth (Gibco), are reported in Figures 4.3, 4.4 and 4.5 respectively. The significance of the effect of the broths on these parameters is recorded in Table 4.10.

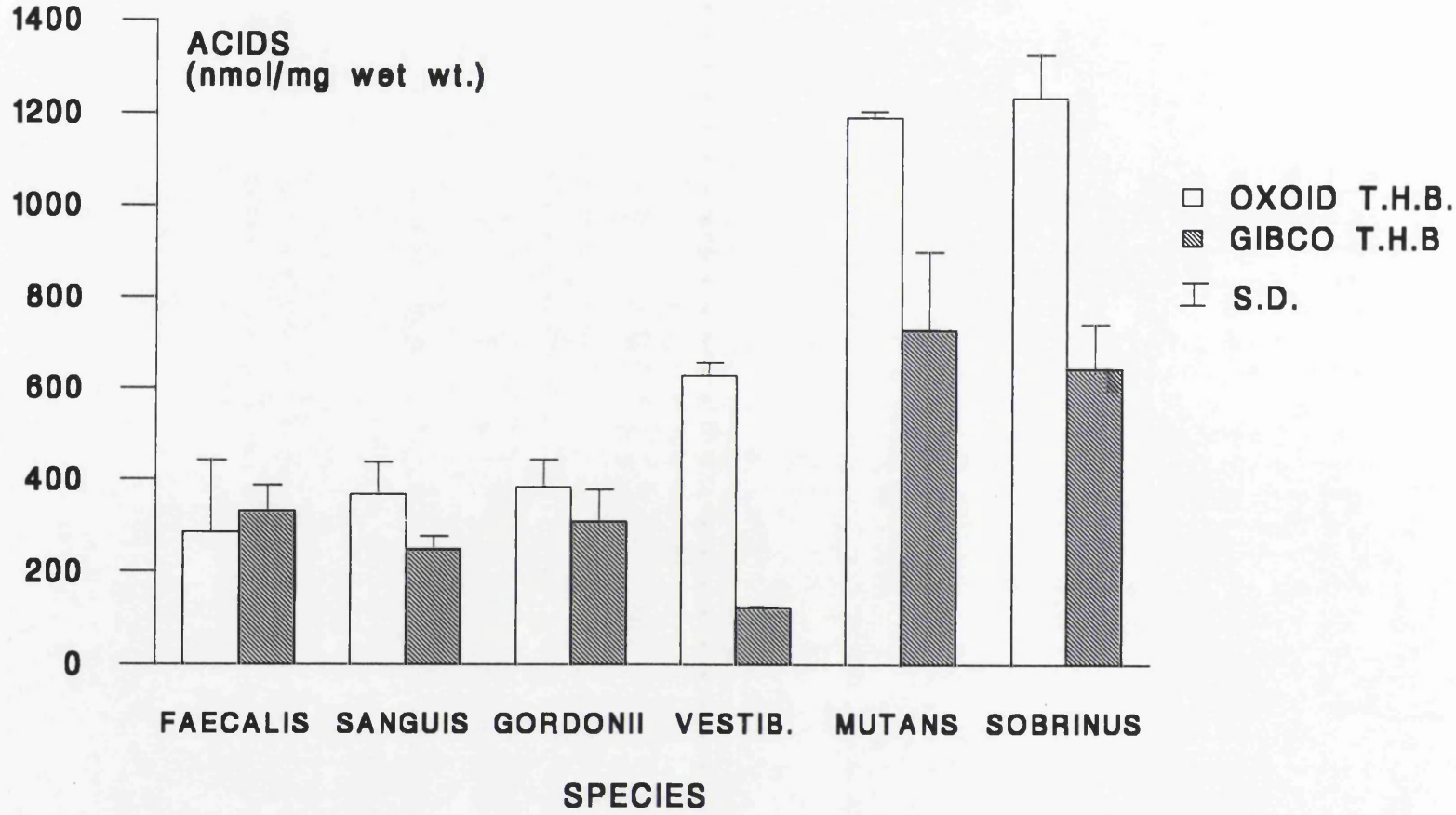


Figure 4.3. Effect of Todd Hewitt Broth on acid production by six species of streptococci.

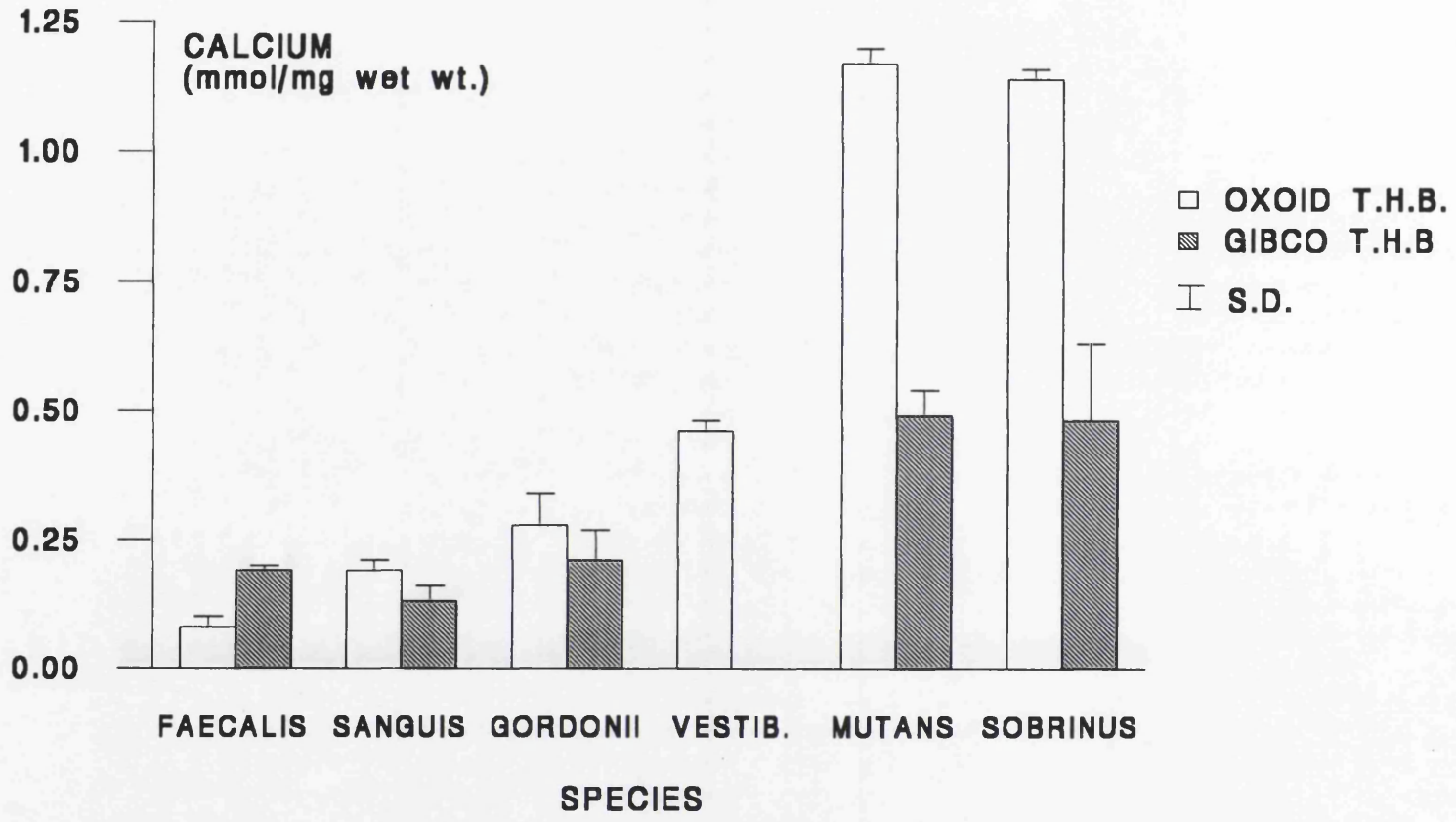


Figure 4.4. Effect of Todd Hewitt Broth on calcium release by six species of streptococci.

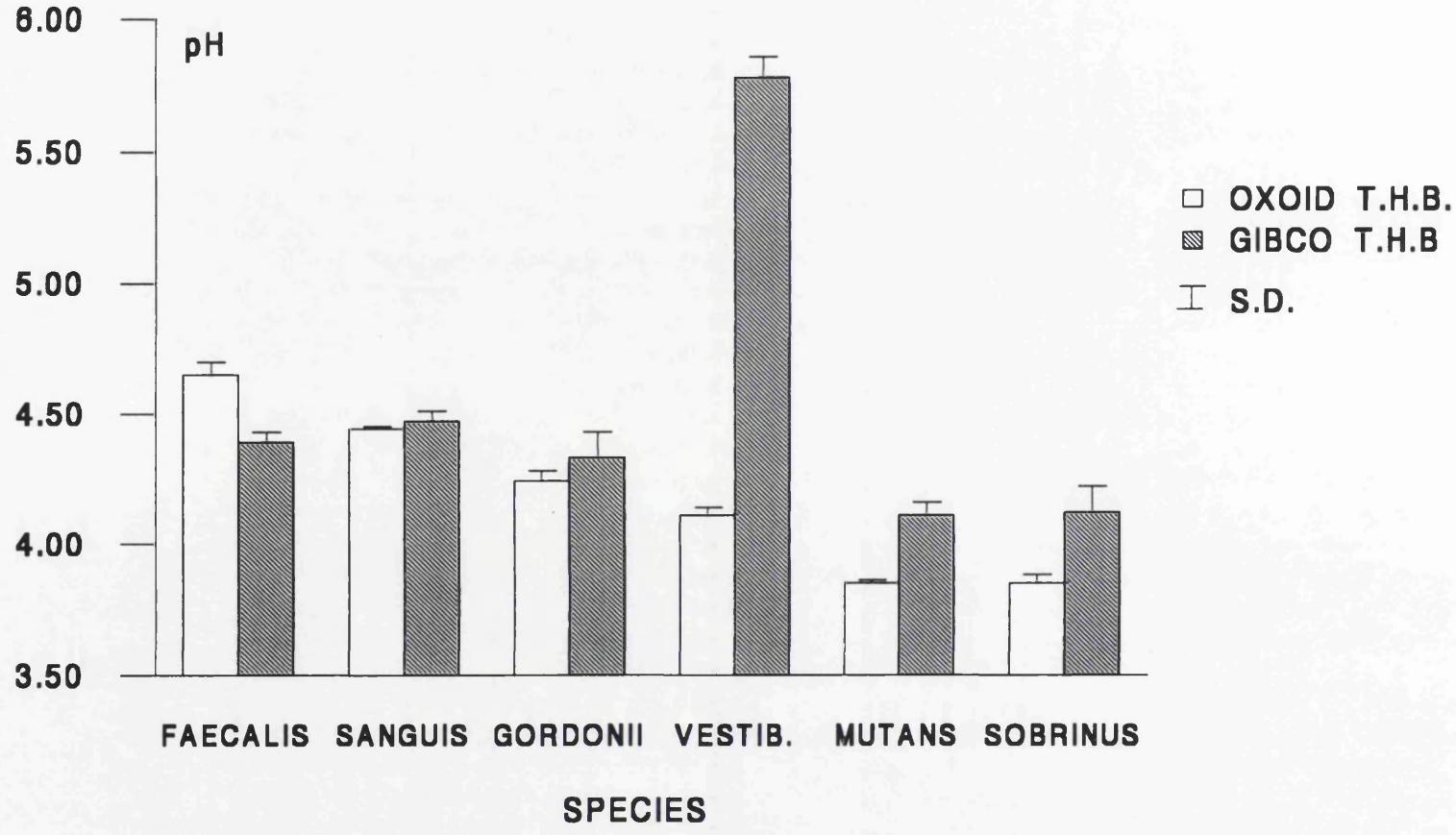


Figure 4.5. Effect of Todd Hewitt Broth on pH following incubation of six species of streptococci.

	Acids	Calcium p values[1]	pH
S. faecalis	NS	0.010	0.005
S. sanquis	NS	0.050	NS
S. gordonii	NS	NS	NS
S. vestibularis	0.001	0.001	0.001
S. mutans	0.050	0.005	0.010
S. sobrinus	0.005	0.010	0.050

[1] = p values assessed by Students' t-tests.

Table 4.10. Significance of differences in acid anion production, calcium release and pH after 5 hours' incubation by Streptococcus species when grown in different Todd Hewitt Broths.

Nutrient broth significantly affected all three determinants of S. sobrinus, S. mutans and S. vestibularis cariogenic potential. For these species, acid production was significantly higher when cells had been grown in the Oxoid manufactured broth. With the exception of S. gordonii, calcium release from hydroxyapatite was significantly higher by all strains when grown in Oxoid broth compared with that supplied by Gibco. Only S. sanguis and S. gordonii failed to demonstrate significantly lower pH values when prepared in the Oxoid broth.

Of particular note are the results obtained with the S. vestibularis strain when incubated in the Gibco manufactured Todd Hewitt Broth. Only minimal acid production was detected during this incubation, no calcium release was observed and the pH fell minimally during the incubation.

4.2.4 Discussion

Kaufman et al., (1984), who studied factors involved in artificial caries induction by oral streptococci in extracted human teeth, showed that in vitro demineralisation could be influenced significantly by the media in which the bacteria were growing. From Appendix A, it is apparent that the composition of the Todd Hewitt Broth supplied by the individual manufacturers used in this study differ in two important respects, namely the

quantity of beef infusion and buffering agents per litre of media.

From the results presented in Figures 4.3 - 4.5, it is apparent that the media effect is most pronounced in those species of highest cariogenic potential. As mentioned above, one possible explanation is differences in the buffering capacity of the broths. Shown in Table 4.11, are the pH values of the Todd Hewitt Broth cultures after 18 hours incubation prior to preparation of the bacterial cells for incubation with the powdered hydroxyapatite. With the exception of S. faecalis, the pH of the culture was significantly lower with the Gibco manufactured broth than that manufactured by Oxoid. Such differences in buffering capacity may affect adversely the viability of cells of low aciduric tolerance. In addition, differences in the nutrient composition of the broths may mean that whilst there was no significant difference in the mean wet weight of cells incubated (39.5 $\pm$ 0.5 mg wet weight with Oxoid and 39.8 $\pm$ 0.5 mg wet weight with Gibco), differences in both nutrient composition and buffering capacity of the Todd Hewitt Broths may have resulted in dissimilar viable cell counts.

In this study, prior to incubation of the bacterial pellet with the hydroxyapatite, a 10 µl sample was removed from the Eppendorf vial after addition of the sucrose solution, and immediately before the

	pH 18 hour culture				
	Oxoid T.H.B.		Gibco T.H.B.		
Species	mean[1]	(S.D.)	mean[1]	(S.D.)	p value[2]
S. faecalis	6.25	(0.02)	6.05	(0.10)	0.070
S. sanquis	6.59	(0.20)	5.89	(0.09)	0.030
S. gordonii	6.66	(0.10)	6.11	(0.03)	0.010
S. vestibularis	6.28	(0.11)	5.63	(0.02)	0.001
S. mutans	6.34	(0.14)	5.63	(0.07)	0.010
S. sobrinus	6.43	(0.07)	5.89	(0.16)	0.030

S.D. = Standard Deviation.

[1] = mean of three replicates

[2] = significance of differences between broths as determined by Students t tests.

Table 4.11 pH of 18 hour cultures of Streptococcus species in Todd Hewitt Broth.

	Log c.f.u./ml incubated				
	Oxoid T.H.B.		Gibco T.H.B.		
Species	mean[1]	(S.D.)	mean[1]	(S.D.)	p value[2]
S. faecalis	10.80	(0.21)	10.78	(0.07)	0.150
S. sanquis	10.28	(0.05)	9.48	(0.25)	0.030
S. gordonii	10.23	(0.31)	9.17	(0.60)	0.110
S. vestibularis	9.42	(0.46)	0.00	(0.00)	0.001
S. mutans	10.24	(0.05)	9.67	(0.14)	0.020
S. sobrinus	9.68	(0.13)	8.42	(0.50)	0.050

Log c.f.u. = Logarithm to the base ten of the colony forming units incubated following incubation in Oxoid and Gibco Todd Hewitt Broths.

S.D. = Standard Deviation.

[1] = mean of three replicates

[2] = significance of differences between broths as determined by Students t tests.

Table 4.12 Effect of different Todd Hewitt Broths on viable cell counts.

hydroxyapatite addition. After appropriate dilution, inoculation on Columbia Blood agar plates and incubation for 2-3 days in a 5% CO_2 incubator, a viable cell count was obtained, and is reported as the logarithm to base ten of the colony forming units per millilitre in Table 4.12.

Whilst there were no significant differences in the weight of cells incubated, with the exception of S. faecalis and S. gordonii, counts were significantly lower with the Gibco-manufactured Todd Hewitt Broth. Of particular interest are the results obtained with the S. vestibularis strain, where viable cells were undetected when cells were cultured in the Gibco Broth which, as will be seen from Table 4.11, produced the joint lowest terminal pH after 18 hours incubation. From these results it is possible that, whilst S. vestibularis has a moderately high acidogenic potential (Table 4.6), the aciduric tolerance of this particular strain is low, and when grown in the more poorly buffered Gibco broth, a bacterial pellet containing very few viable cells resulted. The small quantity of acid detected during the incubation of these cells is attributed to the small number of viable bacteria undetected in the cell counts.

The difficulties encountered in preparing a standardised bacterial pellet for incubation were discussed in detail in Section 4.1.4. Whilst there were no significant

differences in the mean wet weight of bacterial cells incubated, viable cell counts between species ranged from 10.80 (± 0.21) log c.f.u./ml S. faecalis, to 9.42 (±0.46) log c.f.u./ml S. vestibularis.

Technical difficulties were encountered performing the cell counts, especially in producing an entirely homogeneous cell suspension after adding the sucrose solution. It was therefore considered appropriate to follow convention (Marsh et al., 1984; Mayo et al., 1989; De Soet et al., 1989; Watson, Cummins and van der Ouderaa, 1991) and report acid production (and calcium release) on the basis of weight of cells incubated.

4.3 Conclusions

These studies have shown that it was possible to determine the cariogenic potential of six species of streptococcus following the in vitro dissolution of powdered hydroxyapatite, and that the results obtained were in agreement with previously reported cariogenicity of the species concerned. Some of the difficulties encountered in studying metabolic characteristics of microorganisms have been described. Evidence has been provided to show that cariogenic potential, as assessed by this test, may be influenced by the media in which the bacterial cells were prepared.

As discussed in Section 4.1.4, considerable variation

exists in the cariogenicity of individual strains of a bacterial species. Whilst strains considered to be representative of the species were selected for this study, it is not possible to ascribe with certainty cariogenicity to a species, on the basis of results achieved with a single strain.

In view of the current attitude of the general public to animal experiments, this in vitro assay may form a suitable screening test to assess the potential cariogenicity of a range of bacterial species before final testing in an animal model.

CHAPTER 5

THE IN VITRO CARIOGENIC POTENTIAL OF DENTAL PLAQUE.

5.1 An investigation of the relationship between the in vitro cariogenic potential of plaque and in vivo caries experience.

5.1.1 Introduction.

In accordance with Miller's chemico-parasitic theory of dental caries (Miller 1890), it is now generally accepted that colonisation of tooth surfaces by bacteria in the form of dental plaque is a necessary prerequisite for the development of dental caries (Löe, 1969). However, most clinicians are familiar with individuals who in spite of unfavourable oral hygiene, remain caries-free (de Paola et al., 1968; Andlaw, 1978), leading to a poor correlation between caries experience and the various indices of plaque accumulation (Keene, Horton & Handler, 1981).

The study described in this Chapter aimed to investigate the relationship between the in vitro cariogenic potential of dental plaque, determined using the model described in Section 3.4, and clinical caries experience.

5.1.2 Materials and methods.

Immediately following the second clinical examination of the trial described in Section 2.1.1, dental plaque was collected from 51 of the 60 children from whom saliva

samples had been collected at the baseline examination (Section 2.2.2). No specific dietary instructions were issued prior to plaque collection, although each child was asked to confirm that he/she had not eaten within the hour prior to sampling, nor had received antibiotic therapy within the previous six weeks. Plaque was harvested from buccal and labial tooth surfaces, using a sterile plastic instrument (Ash Instruments, Dentsply, Gloucester, England), and placed in a sterile, prelabelled, tared vial. Plaque samples were collected from children with a range of caries experience, but some emphasis was placed on selecting subjects who were either clinically caries-free or who had a high disease experience. The vial was stored on ice and transferred to the microbiology laboratory, Glasgow Dental Hospital where, within three hours, the <u>in vitro</u> cariogenic potential of the plaque was determined.

On arrival in the laboratory, the vial containing the plaque was weighed on a balance which had the ability to measure in increments of 0.01 mg (OHAUS Scale Corporation, New Jersey, USA).

A portion of the plaque (mean weight 2.3 mg) was removed and placed in a bottle containing one millilitre of sterile Anaerobic Blood Broth (Gibco, Paisley, Scotland), to determine viable counts of mutans streptococci. The broth was dispersed using an ultrasonic probe, for 15

seconds at a setting of 1.5 (Heat Systems Ultrasonics, Plainview, USA). The sonicate was then diluted serially, inoculated on Mitis Salivarius Bacitracin Agar and, following appropriate incubation, a viable cell count of mutans streptococci per milligram wet weight of plaque was determined as described previously (Section 2.2).

The original vial containing the remainder of the plaque sample was reweighed. It was therefore possible to determine the total weight of the plaque collected, the weight of the sample used to determine viable cell counts of mutans streptococci, and the weight of the plaque remaining in the vial. The in vitro cariogenic potential of the plaque was then determined, by the method described in Section 3.4, but modified as detailed below.

To the vial containing the plaque (mean weight 11.4 $\pm$1.9 mg), was added 10 mg of powdered hydroxyapatite and 400 µl of a 5% sucrose solution. The vial was then agitated using a vortex mixer for 10 seconds to ensure adequate mixing of the components. The pH of the contents was then recorded immediately (as described in Section 2.9), and the vial incubated for a period of five hours in an orbital shaker (100 rpm) at 37°C. The pH was again measured after 0.5, 1, 2, and 5 hours' incubation. At the end of the incubation period the vials were centrifuged twice at 15000*g* for 20 minutes at 4°C, as described in Section 3.2.3. The resulting supernatant,

free from all debris, was then analysed for acid anion production by isotachophoresis and calcium release by complexation with o-cresolphthalein (Sections 2.3 & 2.6).

5.1.3 Results

The clinical caries experience of the children from whom the plaque samples were collected is recorded in Table 5.1, and is reported as decayed, missing and filled surfaces (DMFS) during the first and third examinations of the clinical trial (Section 2.1). The DMFS values ranged from 0 - 39 during the first examination, and from 0 - 48 during the third examination, mean values being 12.0 ($\pm$11.8) and 16.3 ($\pm$14.8) respectively. Also reported are incremental data between the first and third examinations, the change in DMFS ranging from 0-21 additional surfaces with a mean value of 4.7 ($\pm$4.4).

Salivary counts of mutans streptococci (determined as described in Section 2.2.2), resulted in a mean value of 4.6 ($\pm$1.33) log c.f.u./ml and correlated significantly with caries experience (Table 5.2). This relationship is discussed further in Section 6.1. Mutans streptococci were isolated from 48 of the 51 plaque samples studied, a mean of 4.1 log c.f.u./mg wet weight of plaque being detected. However, no significant relationships were observed between counts of mutans streptococci in the pooled plaque samples, and caries experience (Table 5.2).

	DMFS Range	Mean[a]	(S.D.)
Exam 1	0 - 39	12.0	(11.8)
Exam 3	0 - 48	16.3	(14.8)
Change in DMFS between Exam 1 and Exam 3.	0 - 21	4.7	(4.4)

[a] = Mean of 51 individuals from whom plaque samples were collected.

S.D. = Standard Deviation.

Table 5.1. Caries experience (as DMFS) of the 51 children from whom plaque samples were collected in the study described in Section 5.1.2.

	DMFS at Exam 1	Change in DMFS between Exam 1 and 3
	Correlation coefficient	
log c.f.u. mutans streptococci in saliva[b]	0.47[a]	0.48[a]
log c.f.u. mutans streptococci in plaque	0.06	0.24
Acid anion production	0.08	0.06
Calcium release	0.15	0.03
pH	0.06	0.07

[a] = $p < 0.001$
[b] = determined as described in Section 2.2.2

Table 5.2. Correlation coefficient between acid production, calcium release, pH and mutans streptococci in plaque and saliva and DMFS at Exam 1, and change in DMFS between Exams 1 and 3.

The results of the *in vitro* determination of the cariogenic potential of the plaque samples are presented in Table 5.3. After five hours' incubation, the pH of the vial contents ranged from 4.27 - 5.16 with a mean value of 4.69 (±0.18). Mean acid production at the end of the incubation ranged from 334 - 3121 nmol/mg wet weight of plaque, with a mean value of 1597 (±681). Lactate was again the predominant acid anion detected in the vial supernatant, comprising 75.3 (±9.7)% of total acid production, whilst acetate constituted 8.9 (±5.0)% of acid anions detected. The mean calcium release was 0.63 (±0.26) mmol/mg wet weight, and ranged from 0.09 - 1.23 mmol/mg wet weight plaque.

As shown in Table 5.3 (and indicated diagrammatically in Figure 5.1), significant correlations were observed between acid anion production and calcium release ($r = 0.44$ $p < 0.001$), acid anion production and pH ($r = -0.30$ $p < 0.05$), and calcium release and pH ($r = -0.30$ $p < 0.05$).

Correlations of the determinants of *in vitro* cariogenic potential with caries experience are reported in Table 5.2, from which it can be observed that no significant relationships between either acid anion production, calcium release or pH and DMFS at the first clinical examination, or the change in DMFS between first and third examinations, were detected.

	mean[a]	S.D.
pH	4.69	(0.18)
Acid anions (nmol/mg wet weight plaque)	1597	(681)
Calcium (mmol/mg wet weight plaque)	0.63	(0.26)

	Correlation coefficient	p value <
Acid anions vs Calcium	0.44	0.001
Calcium vs pH	-0.30	0.05
Acid anions vs pH	-0.30	0.05

[a] = mean of 51 plaque samples.

S.D. = Standard Deviation

Table 5.3. The relationship between acid anion production, calcium release and pH after five hours' incubation of 51 plaque samples with powdered hydroxyapatite.

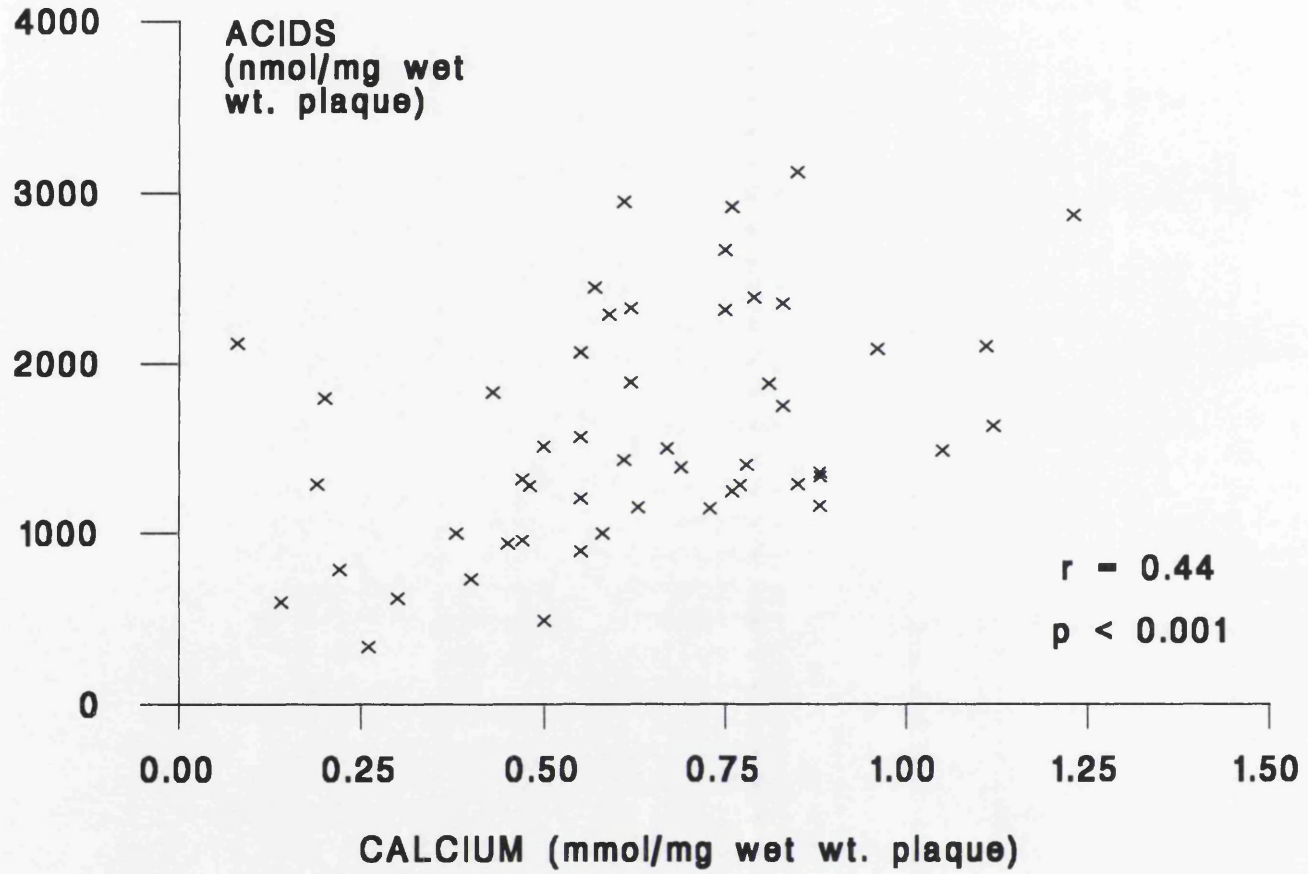


Figure 5.1. Relationship between acid production and calcium release, after 5 hours' incubation of 51 plaque samples with powdered hydroxyapatite.

The relationship between the parameters observed in the in vitro test system was investigated further by comparing results for those children representing the lower and upper quartiles of the baseline DMFS, termed the caries-free and high caries groups respectively (Table 5.4). A similar analysis, which was conducted for the incremental data between examinations one and three, is recorded in Table 5.5. At baseline, upper and lower quartiles were 0 and 23 decayed, missing or filled surfaces, the corresponding DMFS values for the incremental data being 2 and 7 respectively.

As can be noted from Table 5.4, highly significant differences were observable in the salivary counts of mutans streptococci between high caries and caries-free groups. However, whilst higher mean acid production and calcium release, and lower pH values, were observed when the high caries group was compared to the clinically caries-free children, such differences did not prove to be statistically significant. The percentage of lactate comprising total acid production was also higher in the high caries group, but again did not differ significantly from the caries-free group.

When the data relating to those children who experienced a caries increment of two surfaces or less were compared to those whose decayed missing or filled scores increased by seven or more (Table 5.5), only salivary counts of

	DMFS at Exam 1	
	Caries-Free	High Caries (DMFS ≥ 23)
	mean[a] (S.D.)	mean[a] (S.D.)
Salivary counts of mutans streptococci (log c.f.u./ml)	3.57 (1.49)	5.14 (0.98)[1]
Plaque counts of mutans streptococci (log c.f.u./ml)	4.30 (1.61)	4.35 (1.28)
Calcium (mmol/mg wet weight plaque)	0.62 (0.22)	0.72 (0.25)
Acid anions (nmol/mg wet weight plaque)	1363 (498)	1498 (611)
Percentage of		
(a) lactate	74.2 (9.7)	76.6 (7.7)
(b) acetate	8.0 (4.3)	9.0 (5.8)
comprising total acid production		
pH	4.75 (0.20)	4.67 (0.20)

[a] = mean of 16 individuals
[1] = significantly different ($p < 0.001$) as assessed by Students t-test.
S.D. = Standard Deviation

Table 5.4. Differences in salivary counts of mutans streptococci and <u>in vitro</u> determinants of the cariogenic potential of dental plaque collected from caries-free and high caries groups, based on DMFS at baseline examination.

	Change in DMFS between Exam 1 and Exam 3	
	≤ 2 surfaces	≥ 7 surfaces
	mean[a] (S.D.)	mean[b] (S.D.)
Salivary counts of mutans streptococci (log c.f.u./ml)	3.80 (1.36)	5.38 (0.91)[1]
Plaque counts of mutans streptococci (log c.f.u./ml)	4.23 (1.33)	3.83 (1.71)
Calcium (mmol/mg wet weight plaque)	0.62 (0.26)	0.63 (0.32)
Acid anions (nmol/mg wet weight plaque)	1503 (652)	1613 (769)
Percentage of lactate comprising total acid production	73.4 (8.72)	72.3 (12.3)
pH	4.78 (0.16)	4.79 (0.28)

[a] = mean of 18 individuals
[b] = mean of 14 individuals
[1] = significantly different ($p < 0.001$) as assessed by Students t-test.
S.D. = Standard Deviation

Table 5.5. Differences in salivary counts of mutans streptococci and <u>in vitro</u> determinants of the cariogenic potential of dental plaque collected from individuals experiencing caries increments ≤ 2 and ≥ 7 surfaces, between the first and third examination.

mutans streptococci differed significantly between groups.

5.1.4 Discussion.

The in vitro model developed as described in Section 3.4, had been shown to be capable of differentiating streptococcal species on the basis of acid anion production, calcium release and changes in pH during incubation of powdered hydroxyapatite in the presence of a sucrose solution (Chapter 4). The study described in this Chapter was therefore conducted to determine if the model could be utilised to identify differences in dental plaque collected under "field conditions".

Before discussing the results of the study in full, the method of diagnosing and recording the caries experience of children from whom the plaque samples were collected requires explanation.

Caries was recorded on the basis of a clinical oral examination which included fibre optic transillumination of the interproximal surfaces. It was therefore possible that those children reported as clinically caries-free, may have had early interproximal lesions which remained undetected by the examination techniques employed. However, it seems unlikely that any resulting under-diagnosis would affect the overall results of the current study. Caries was reported on the basis of decayed,

missing and filled surfaces, with third permanent molars being excluded. In a novel approach, caries in the pits of the palatal surfaces of upper permanent molars, and incisors, and in the buccal surfaces of lower molars was recorded separately from smooth caries on the same surfaces. There were, therefore, a total of 144 surfaces at risk, as opposed to the conventional 128 surfaces. Surfaces exhibiting defects in enamel morphology were excluded from analysis, as were teeth congenitally absent or lost due to either trauma or orthodontic extraction. In the analysis of the caries increment, no account was taken of the possible effect of the dentifrice, due to the small numbers involved and the exclusion of initially caries-free children from the trial. However, participants were evenly distributed across the six product groups being tested and, as the incremental data is reported after two years, it was thought unlikely that inclusion of these parameters would have significantly influenced the results of the current study.

In a now famous experiment, Stephan (1944) investigated the <u>in vivo</u> pH response of dental plaque after rinsing with a carbohydrate solution, and reported that "resting" pH values, as well as the lowest pH obtained, were related to caries activity. In the interim, numerous studies have examined factors influencing the cariogenic potential of dental plaque. These investigations can be broadly differentiated into those which pursued the

relationship between caries and the microbial composition of plaque, and those which studied biochemical properties of plaque, expressed either as pH changes following exposure to carbohydrate, or as the composition of resting or starved plaque fluid.

The relationship between the microbial composition of plaque and caries experience is considered first. Whilst a significant correlation between salivary counts of mutans streptococci and dental caries has been reported by numerous investigators (Klock & Krasse, 1977; Arneberg et al., 1984; Wilson & Ashley, 1989; Salonen et al., 1990, & Russell et al., 1991), findings which were supported by the current study, there are few reports in the literature of a similar clear-cut relationship between mutans streptococci in pooled plaque samples and overall caries experience, which again agrees with the current investigation. In a study of the proportions of S. mutans isolated from pooled plaque samples harvested from a total of 95 caries-free and caries-prone dental students, Hayes, Carter and Griffiths (1983) reported that although the caries-prone group included those subjects with the greatest proportions of S. mutans, they did not differ significantly from the caries-free group.

The lack of a clear-cut relationship between mutans streptococci in pooled plaque and caries, is perhaps somewhat surprising, as a number of studies (Togelius et

al., 1984; Keene, 1986; Schaeken, Creugers & van der Hoeven, 1987 & Mundorff et al., 1990), have reported positive correlations between levels of mutans streptococci in saliva and plaque. Salonen et al. (1990), investigated the relationship between salivary levels of mutans streptococci, oral hygiene and caries in an adult Swedish population, and reported that whilst a consistently higher mean number of decayed surfaces was observed among the subjects with higher numbers of mutans streptococci, this bore no relation to plaque scores, and that no consistent relationship was observed between plaque scores and mean number of decayed surfaces when individuals with similar numbers of salivary mutans streptococci were compared.

There have, of course, been numerous studies which have demonstrated significant associations between mutans streptococci and caries at specific tooth sites (Hoerman et al., 1972; Keene, Horton & Handler 1981; Milnes & Bowden, 1985; van Houte et al., 1991a). However, the failure to demonstrate significant relationships between the numbers of mutans streptococci in pooled plaque and caries may be due to the highly localised nature of the carious lesion, and the associated cariogenic plaque. Furthermore, in their study, van Houte and co-workers (1991a) again raised the question of the contribution of bacteria other than mutans streptococci to the acidogenic potential of plaque.

The inadequacies of the DMFS score in quantifying decay, have been discussed by Loesche (1986), and are evidenced in this study by the finding that plaque mutans streptococci counts, and the incremental data, were more closely related than was observed with the baseline DMFS (Table 5.2), although not significantly so ($p < 0.10$).

The *in vitro* incubation of plaque as a means of investigating pH changes in plaque following exposure to carbohydrate, has been known for many years. In the current study, the mean pH of the vial contents after five hours' incubation was 4.69 ($\pm$0.18), and ranged from 4.27 - 5.16, values that are within the range reported by Geddes (1972), who had also incubated plaque samples *in vitro* with a 5% sucrose solution. These values are also similar to those reported by Igarashi, Lee and Schachtelle (1990) who, in studying sucrose-induced plaque pH profiles in artificial fissures *in vivo*, reported pH minima of 4.8 ($\pm$0.2), 4.6 $\pm$0.2 and 5.0 ($\pm$0.3) in one, two and four-day-old plaque respectively. In the current study, no attempt was made to standardise the age of the plaque sample collected and, even although these teenagers were participating in a dentifrice-related oral health trial, it is not unlikely that a portion of the plaque collected had been present for more than four days.

A large range of values for total acid anion production

was observed and, interestingly, the mean acid production per milligram wet weight of plaque was higher than that observed with pure cultures of Streptococcus species observed in the study described in Chapter 4. However the weight of plaque incubated in this study was lower, by a factor of four, and it is possible that pure cultures of streptococci require to produce less acid per milligram before inhibitory pH levels are encountered. Equally, other factors such as the buffering effect of plaque constituents, and the interaction of different bacterial species, are likely to have influenced acid production.

Geddes et al. (1984), have commented on the importance of acid type and concentration, rather than pH alone, in determining demineralisation potential. In the current study, lactate was the major acid anion present, which is in agreement with the study described in Chapter 4, and with the work of Geddes et al. (1984). Whilst lactate constituted a greater proportion of total acid production in the high caries group, this was not significantly different from the caries-free group.

A number of previous workers have investigated the relationship between organic acids in plaque and caries susceptibility. However, the methodology employed differed from that of the current study, making direct comparison difficult. Vratsanos and Mandel (1982),

claimed that, when ten caries-resistant and ten caries-susceptible subjects chewed a sucrose-containing gum for 45 minutes, both the amount and rate of lactate production *in vivo* were lower, and the amount of acetic acid higher, in the caries-resistant group. This led them to propose that high pK_a acids, such as acetic, can provide a buffering system capable of countering the pH decrement generated by low pK_a acids such as lactic. However, closer examination of the results of Vratsanos and Mandel (1982), show that at 45 minutes, neither the amount of lactate nor total acid production differed significantly between caries-susceptible and caries-resistant groups. Furthermore, in their study the plaque had been collected in a highly standardised fashion, subjects being given a prophylaxis prior to participation in the study, and strict dietary and oral hygiene protocols were employed prior to plaque collection. A more recent study by Margolis, Duckworth and Moreno (1988a), investigating the composition of pooled resting plaque samples (again collected in a highly standardised fashion), failed to demonstrate significant differences in the concentration of high pK_a and low pK_a acids or in the total concentration of acid anions between caries-free and caries-susceptible individuals. This was in spite of previous computer predictions by the same group (Margolis, Moreno & Murphy 1985), which supported the theory of Vratsanos and Mandel (1982). Margolis *et al*. (1988a) commented that, although the composition of

resting plaque fluid may be important to the overall caries process, their results did not preclude the possibility that following exposure to fermentable carbohydrate, differences in organic acid composition would become apparent and correlate with caries susceptibility. Whilst the current study did not investigate plaque fluid, nor did it employ standardised plaque samples, no clear relationship between either the proportion of lactate, acetate or total acid anion production and caries susceptibility could be demonstrated following exposure of the plaque to the sucrose solution.

It is concluded, therefore, that whilst it may be possible to demonstrate significant differences in plaque fluid which relate to caries experience, such differences may be in evidence only when the plaque samples have been collected in a highly standardised fashion, and from individuals with extremes of caries experience (Margolis, 1990). As evidenced by the results of the current study, it is much more difficult to demonstrate inherent differences in pooled plaque samples collected under field conditions, and to relate these to caries experience. An explanation for this situation has recently been offered by Tanzer (1989), who criticised the study of pooled plaque stating, that because of the highly localised nature of caries lesions, studies should focus on plaque associated with such lesions, due to the

uncertain composition and relation of pooled plaque to the underlying enamel.

It is apparent, therefore, that whilst the in vitro model described in Section 3.4 had been used to significantly differentiate species of streptococci (see Chapter 4), it was not possible to relate the in vitro cariogenic potential of plaque to clinical disease experience.

CHAPTER 6

PRELIMINARY STUDIES ON THE CARIOGENIC POTENTIAL OF STREPTOCOCCUS MUTANS.

6.1 Salivary counts of mutans streptococci, Lactobacillus species and salivary flow rates.

6.1.1 Introduction.

Significant relationships between salivary counts of Lactobacillus species and caries experience and of mutans streptococci and caries were first reported by Rodriguez (1931) and by Krasse et al. (1968) respectively, and in the interim, numerous studies have repeated these findings (Ellen, 1976; Beighton, 1991). The role of salivary flow-rates in determining caries risk was discussed in Section 1.17.2.

As described in Section 2.2, mutans streptococci and Lactobacillus species were isolated and salivary flow-rates determined in the course of a clinical dentifrice trial. This section records the relationship of the microbiological and clinical parameters investigated, as well as the caries experience of the children from whom the samples were collected.

6.1.2 Materials and methods.

Here, 60 children, with a range of caries experience, were selected and saliva samples collected as described in Section 2.2.1. A total of 271 strains of mutans

streptococci and 182 strains of Lactobacillus species were isolated, enumerated and stored for future study (Section 2.2). Participants in the clinical trial were identified by a four digit number which was also used to label the bacterial isolates. Up to 10 strains of mutans streptococci were isolated from each individual and were labelled using the identification number suffixed by the letters A-J. Lactobacillus species were similarly identified, suffixed by the letters S-Z.

Statistical analysis was conducted as described in Section 2.4.2. In view of the skewed distribution of the microbiological counts, they were converted to decimal logarithms prior to statistical analysis. In those cases where mutans streptococci or Lactobacillus species remained undetected, a count one half of the minimum detection value was assigned (Russell et al., 1990) to allow the logarithmic conversion of zero counts.

6.1.3 Results.

Salivary counts of mutans streptococci ranged from 1×10^3 to 6.93×10^6 c.f.u./ml, and were zero for four individuals. Counts of Lactobacillus species ranged from under 6×10^2 to 1.6×10^5 c.f.u./ml, with five individuals having zero counts. As recorded in Table 6.1 and Figure 6.1, significant correlations were observed between salivary counts of mutans streptococci and DMFS or DMFT, ($r=0.443$, $p < 0.001$; & $r=0.465$, $p < 0.001$)

	Correlation coefficient	p value <
DMFS vs mutans streptococci	0.443	0.001
DMFT vs mutans streptococci	0.465	0.001
DMFS vs Lactobacillus species	0.489	0.001
DMFT vs Lactobacillus species	0.505	0.001
DMFS vs salivary flow-rate	-0.088	NS
DMFT vs salivary flow-rate	-0.044	NS

NS = not significant

DMFT & DMFS at exam 1

Table 6.1. Relationship between DMFS, DMFT and salivary counts of mutans streptococci, Lactobacillus species and salivary flow-rates.

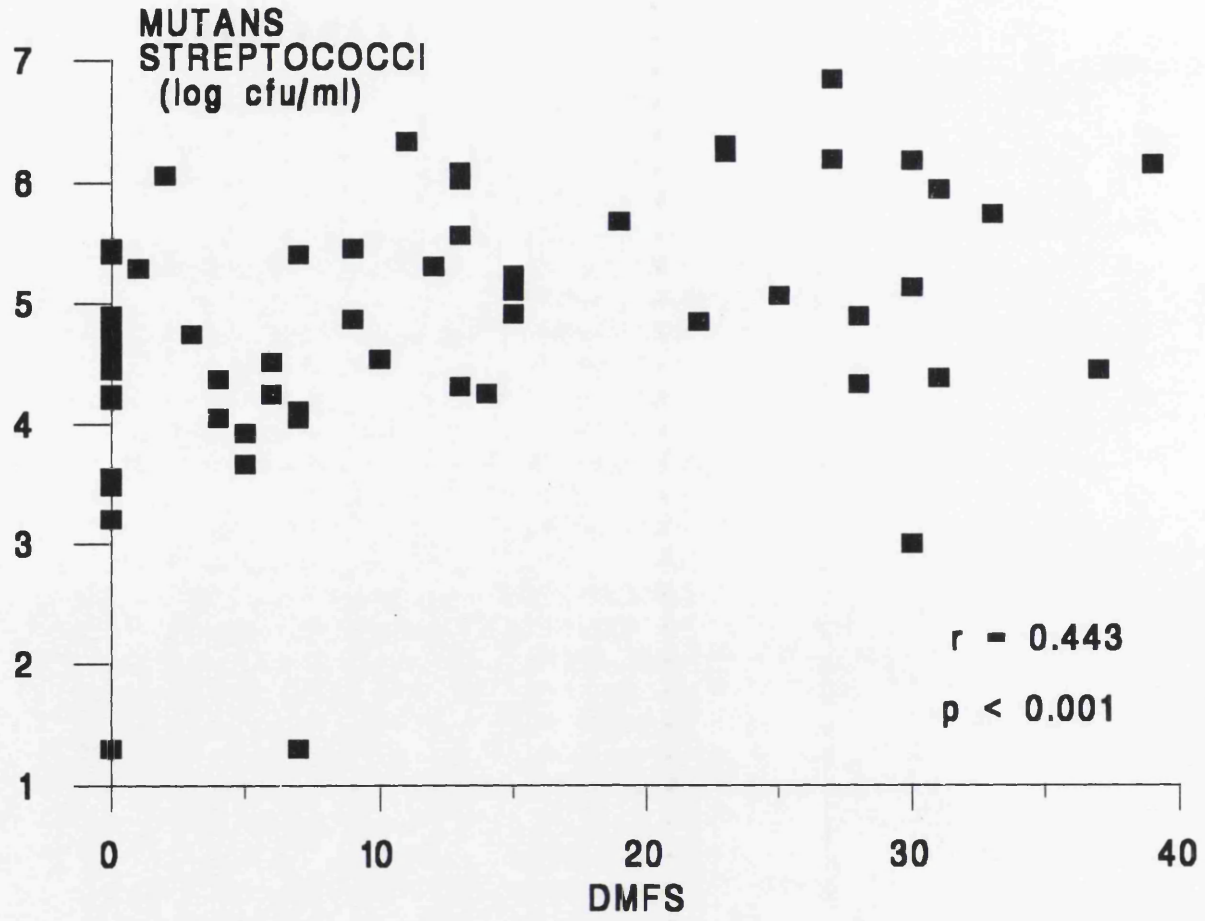


Figure 6.1. Correlation of DMFS and salivary count of mutans streptococci.

respectively. Similar associations were observed between DMFS and Lactobacillus species ($r=0.489$, $p < 0.001$) and DMFT and Lactobacillus species ($r=0.505$, $p < 0.001$). However no significant correlations were observed between DMFS or DMFT and salivary flow-rates.

6.1.4 Discussion.

The failure to demonstrate significant correlations between salivary flow-rates and caries experience in this study are in agreement with the findings of Klock and Krasse (1979). The relationship of salivary flow-rates and caries has been extensively investigated (Pearce 1991), but, except in cases of underlying pathology e.g. xerostomia (Dreizen & Brown 1976), the significance of such a relationship is unclear. This is perhaps not surprising since it has been shown that, even with what might be regarded as normal flow-rates, a ten fold difference in both stimulated and unstimulated salivary flow is possible (Ferguson, 1975).

Significant correlations between salivary counts of mutans streptococci and Lactobacillus species have been reported by many investigators (Krasse, 1985; Emilson & Krasse, 1985; Kingman et al., 1988, Weinberger & Wright, 1989; and Alaluusua et al., 1990). Russell et al., (1990 & 1991) investigated microbiological and salivary caries activity tests in a similar population to that currently studied. The shortcomings of present caries activity

tests in identifying the high risk individual were highlighted by that study and have been discussed previously (Section 1.18). In this investigation, whilst organisms were isolated from children with a range of caries prevalence, emphasis was placed on collecting samples from those at either extreme of the disease experience range. This may account for the relatively high correlation coefficients observed. Whilst the relationship between caries experience and salivary counts of microorganisms is widely reported in the literature, usually in the form of a table giving the correlation coefficient, such data is seldom presented graphically, (see Figure 6.1). In this Figure, individuals can be readily identified who, in spite of having a high count of mutans streptococci, remain clinically caries free. Conversely there exist individuals with high disease experience harbouring only moderate counts of mutans streptococci. These results highlight the question of central importance to this thesis, i.e. can the disparity between caries experience and salivary counts, be wholly or partly explained by differences in the cariogenic potential of the mutans streptococci harboured by individuals?

6.2 Preliminary study of the cariogenic potential of twenty strains of S. mutans.

6.2.1 Introduction and aims.

Macpherson (1988) and Macpherson et al., (1992) claimed

that a significant relationship existed between caries experience and the *in vitro* cariogenic potential of six adult subjects (see Section 3.2). Section 3.4 described an *in vitro* model, developed to determine rapidly the cariogenic potential of bacterial isolates. This model involved dissolution of powdered hydroxyapatite during incubation with a bacterial slurry in a sucrose solution. Acid anion production, calcium release and changes in pH during incubation, were assessed as determinants of cariogenic potential. Hence the present study aimed to further investigate the relationship between caries experience, as expressed by DMFS, and *in vitro* cariogenic potential.

6.2.2 Materials and Methods.

The cariogenic potential of 20 strains of *S. mutans* was investigated. These strains were isolated from 20 children, nine of whom were clinically caries-free, and 11 of whom had a DMFS > 23, subsequently called the "caries-free" and "high caries" groups. The organisms were studied in four experimental batches; strains from the caries-free and high caries groups being randomly distributed throughout. In each batch, six strains were investigated with the Type strain *S. mutans* NCTC 10449 being included to act as a control. These children's salivary mutans streptococci counts had been determined previously (see Section 6.1).

Statistical analysis was conducted as described in Section 2.4.2.

6.2.3 Results.

The mutans streptococci salivary counts of the 20 children investigated in this study, determined as described in Section 2.2.1, are recorded in Table 6.2. By Mann-Whitney U tests (Section 2.4.2), a significant difference ($p < 0.02$) was observed between the mutans streptococci salivary counts of the caries free and high caries group. The cariogenic potential of the 20 strains of S. mutans, as defined by acid anion production, calcium release and pH during incubation with powdered hydroxyapatite, is recorded in Table 6.3. Mean calcium release by strains isolated from the high caries group was higher than that released by strains isolated from the caries-free group (13.66 mmol/l compared with 10.70 mmol/l), although the difference was not statistically significant. Noteworthy are the data relating to calcium release by strains 1975A and 5595A in the caries-free group, where calcium data was approximately double the mean calcium release for the group.

Mean acid production was also higher in the high caries group, (455 nmol/mg wet weight bacteria), compared to the caries-free group (331 nmol/mg wet weight). However the large range of values found within each group resulted in no statistically significant differences in acid anion production. No significant differences were observed in

CARIES-FREE GROUP		HIGH CARIES GROUP	
Individual	mutans strep.[a]	Individual	mutans strep.[a]
1879	5.45	1942	6.19
1975	4.60	3620	5.18
3587	4.07	4367	6.17
4351	3.47	4445	4.89
5045	4.45	5227	6.84
5595	4.90	5359	5.73
6421	4.62	5666	4.44
7289	3.78	5725	6.31
8401	5.40	5777	6.14
		6201	5.13
		6303	4.33
Mean	4.53	Mean	5.58[b]
S.D.	0.68	S.D.	0.83

[a] = log.cfu/ml saliva.
[b] $p < 0.02$ as assessed by Mann-Whitney U test.
S.D. = Standard Deviation.

Table 6.2. Salivary counts of mutans streptococci by low and high caries prevalence.

(a) CARIES FREE GROUP

Strain	Calcium[a]	Acids[b]	pH
1879A	2.63	145	4.86
1975A	21.28	691	4.26
3587A	6.25	215	3.95
4351B	9.45	251	4.52
5045A	9.20	322	4.51
5595A	22.30	503	4.38
6421A	6.36	240	4.60
7289A	9.55	350	4.67
8401A	9.28	259	4.60
mean	10.70	331	4.48
S.D.	6.68	169	0.26

[a] = mmol/l.
[b] = nmol/mg wet wt. bacteria.
S.D. = Standard Deviation.

(b) HIGH CARIES GROUP

Strain	Calcium[a]	Acids[b]	pH
1942C	7.00	266	4.70
3620A	8.72	270	4.70
4367H	11.11	268	4.52
4445A	14.36	380	4.53
5227A	10.27	605	4.33
5359A	14.06	595	4.53
5666A	14.03	468	4.45
5725A	9.66	344	4.56
5777A	34.13	852	4.41
6210A	15.72	715	4.43
6303A	11.23	239	4.61
Mean	13.66	455	4.52
S.D.	7.30	209	0.11

[a] = mmol/l.
[b] = nmol/mg wet wt. bacteria.
S.D. = Standard Deviation.

Table 6.3. Calcium release, acid production and pH following incubation with powdered hydroxyapatite, by (a) caries-free, or (b) high caries status.

the pH of the vials after 5 hours' incubation.

Results obtained with control strain S. mutans NCTC 10449 for acid anions, calcium release and pH were 325 (±60), nmol/mg wet weight bacteria, 11.57 (±1.97) mmol/l and 4.50 (±0.11) respectively.

6.2.4 Discussion.

This preliminary experiment aimed to investigate the in vitro cariogenic potential of 20 strains of S. mutans using the test devised in Section 3.4. It was hoped that, by selecting strains of S. mutans isolated from children at either end of the spectrum of disease activity, significant differences in in vitro cariogenic potential would be observed. As expected, significant differences were recorded in salivary counts of mutans streptococci between the caries-free and high caries groups (Table 6.2). However, whilst higher mean acid production and calcium release were observed in the high caries group, these were not significantly different from the low caries group.

Calcium results are reported as the total calcium concentration (mmol/l) of the vial supernatant following incubation. While the aim of the experiment was to incubate 40 mg of bacterial cells, achieving a bacterial pellet of exactly 40 mg wet weight was technically difficult (see Section 4.1.4). Thus the mean weight

incubated in this series of experiments was 39.3(±1.5)mg for the caries-free strains and 39.4(±2.4)mg for the high caries group. If the calcium release data are expressed as mmol/mg wet weight of bacteria, a mean calcium release of 0.27(±0.17)mmol/mg for the caries-free strains, and 0.35(±0.19)mmol/mg for the high caries strains is produced. Statistical analysis of this data still proved to be not significant.

Therefore a clear difference in cariogenic potential between those strains isolated from the caries-free children and from those in the high caries group was not demonstrable. Of note were the particularly high values obtained with strains 1957A and 5595A in the caries-free group. In this preliminary study, strains were tested on only one occasion and therefore five strains were selected for further study as reported in the following Section 6.3.

6.3 Further studies on the cariogenic potential of *S. mutans*.

6.3.1 Introduction and aims.

This study aimed to investigate, in greater detail, the *in vitro* cariogenic potential of five strains of *S. mutans* studied previously in Section 6.2.

6.3.2 Materials and Methods.

The cariogenic potential of five representative strains

(1879A, 6421A, 6303A, 5359A & 5777A) was investigated using the model developed in Section 3.4. The strains were selected for their ability to produce a spectrum of calcium release values as described in Section 6.2. Each strain was tested on four separate occasions, a new culture of each organism being retrieved from Protect bead storage on each occasion. S. mutans NCTC 10449 was incubated to act as a reference strain and was included in each experimental batch.

Statistical analysis was conducted as discussed in Section 2.4.2.

6.3.3 Results.

Calcium release, acid anion production and pH data after 5 hours' incubation with powdered hydroxyapatite are reported in Table 6.4. In agreement with the findings in Section 6.2.3, the lowest and highest calcium release values were obtained with strains 1879A and 5777A respectively. Differences in calcium release between strains, as assessed by analysis of variance, were statistically significant ($p < 0.001$). By 95% Scheffé tests, strain 5777A was significantly different from all other strains except 10449, and strain 10449 was significantly different from strain 1879A. Additionally, significant discrimination between strains was also possible on the basis of acid production ($p < 0.002$) and minimum pH ($p < 0.001$), as reported in Table 6.4.

Strain	1879A	6421A	6303A	5359A	5777A	10449
Calcium mmol/l						
mean	2.06	6.52	6.65	6.93	17.37	11.88
S.D.	0.90	1.93	2.22	2.08	4.16	3.61
Acid anions nmol/mg						
mean	164	210	249	229	387	289
S.D.	31	47	72	67	88	47
min pH						
mean	5.01	4.69	4.69	4.69	4.44	4.54
S.D.	0.16	0.05	0.08	0.05	0.10	0.03

mean = mean of four determinations.
S.D. = Standard Deviation

Significance of differences between strains as assessed by analysis of variance.

	Calcium	Acid	pH
Degrees of freedom	5, 18	5, 18	5, 18
F ratio	15.30	6.20	16.85
p value	0.001	0.002	0.001

Significant differences by 95% Scheffé Tests.

Calcium	5777A vs 1879A, 6421A 5359A and 6303A
	10449 vs 1879A.
Acids	5777A vs 1879A and 6421A
pH	5777A vs 1879A, 6421A and 6303A.
	10449 vs 1879A

Table 6.4. Calcium release, acid production and pH following incubation of strains 1879A, 6421A, 6303A, 5777A and 10449.

Acid production has been reported as the total of the specific acid anions detected by isotachophoresis. Table 6.5 records the proportion of the major specific acid anions detected. It is apparent that under the conditions prevailing in this test, lactic acid forms the major acid end-product. The amount of lactic acid as a proportion of total acid anions ranged from 75.9 (±9.9)% by strain 6303A to 87.7 (±4.1)% by strain 5777A. Acetate comprised the second most abundantly produced anion comprising from 7.7 (±2)% to 13.9 (±11.5)% by strains 5777A and 6303A respectively. The remainder of acid production consisted of formate, pyruvate, succinate and propionate, these usually comprising less than 10% of total acid production. In spite of differences in the total amount of acids produced by different strains, no significant differences in the proportion of lactate or acetate produced by the strains were observed.

Data in Table 6.6 report phosphate release which ranged from 103 (S.D.±11) nmol/mg wet weight bacteria by strain 1879A, to 118 (S.D.±8) nmol/mg wet weight bacteria by strain 5777A. Whilst significant correlations between phosphate and total acid anion production, calcium release and pH after 5 hours' incubation were observed, it was not possible to discriminate significantly between the strains on the basis of phosphate release.

Strain	1879A	6421A	6303A	5359A	5777A	10449
Lactate						
mean	79.1	81.9	75.9	82.0	87.7	84.7
S.D.	8.6	3.7	9.9	4.8	4.1	4.0
Acetate						
mean	9.1	8.8	13.9	8.9	7.7	7.9
S.D.	3.9	1.9	11.5	3.7	2.0	3.3
Others						
mean	11.8	9.9	10.1	8.8	4.5	7.3
S.D.	4.8	4.9	3.8	5.5	2.2	1.7

mean = mean of four determinations
S.D. = Standard Deviation

Table 6.5. Specific acid anions as a percentage of total acid anion production.

Strain	1879A	6421A	6303A	5359A	5777A	10449
Phosphate nmol/mg wet weight						
mean	103	104	110	106	118	114
S.D.	11	16	7	17	8	9

	Correlation Coefficient	p value
Phosphate vs Calcium	0.942	0.01
Phosphate vs Acids	0.965	0.001
Phosphate vs pH	0.851	0.02

mean = mean of four determinations.
S.D. = Standard Deviation

Table 6.6. Relationship of phosphate anions and calcium release, acid production and pH following incubation of strains 1879A, 6421A, 6303A, 5777A and 10449.

6.3.4 Discussion

In this study it was again possible to demonstrate significant differences in the *in vitro* cariogenic potential of *S. mutans* strains.

Considering firstly acid anion production, the results of the current study are in agreement with those of Robrish and Krichevsky (1972) who reported that lactate comprised 85% of acid production by cultures of *S. mutans*.
It has been reported that acid anions differ in their demineralising potential (Featherstone & Rodgers, 1981; Patel, *et al*. 1987). It may be, therefore, that the cariogenic potential of a strain is influenced, not only by the total acid anions produced, but by the proportion of the different acid anions comprising total acid production. Whilst this experiment resulted in differences in total acid production by different strains, no significant differences (as assessed by analysis of variance) in the proportion of specific acid anions comprising total acid production were observed.

The metabolic end-products of carbohydrate fermentation have, however, been reported to be strongly influenced by the prevailing environmental conditions (Carlsson, 1986; Marsh & Keevil, 1986) and, in particular, whether the carbohydrate is present in excess. Reynolds and Riley (1981) have shown that maximal sucrose uptake occurs at a sucrose concentration of 2 mM, which is exceeded by

the conditions employed in this study. If the sucrose available for fermentation had been limited or supplied at a concentration sufficient to stimulate glycolysis by the lactate dehydrogenase pathway in some strains but not in others (i.e. if differences exist between strains in the threshold at which the "lactate gate" is opened), it may have been possible to demonstrate differences in the proportion of acid anions produced by different strains.

The finding that phosphate production correlated with calcium release (Table 6.6) is in agreement with the findings of ten Cate and Duijsters (1983) who reported such a relationship during the dissolution of dental enamel. However no significant differences in phosphate production by the strains investigated were noted, in spite of significant differences being observed in calcium release, acid anion production and pH after 5 hours incubation (Tables 6.4 & 6.6).

Isotachophoresis detects phosphate only in the ionized form, and it is likely that phosphate acts as a buffer in this system (see Chapter 8), thereby remaining undetected. Luoma (1980) proposed that bacterial cells act as a "phosphate reservoir" and he showed that during sucrose fermentation, the uptake of ^{32}P from bovine enamel powder by S. mutans cells was higher than by control cells. The capacity of S. mutans to take up and accumulate environmental phosphorous may be very high

(Tanzer & Krichevsky, 1970). If Luoma's theory that the bacterial cells act as an intracellular reservoir is correct, then the failure to discriminate significantly between strains may be due to phosphate uptake by the cells.

Therefore for both reasons (i.e. the buffering effect and bacterial uptake), phosphate release from the hydroxyapatite was not judged suitable as a determinant of cariogenic potential in this model. Furthermore, whilst as described in Section 2.3, it was possible to detect phosphate by isotachophoresis, in accordance with the method described by Geddes and Weetman (1981), throughout this thesis phosphate was excluded from reported total anion production.

From the studies described in this Chapter, it is apparent that differences in the *in vitro* cariogenic potential of selected strains of *S. mutans* could again be demonstrated (see Chapter 3). However in this preliminary study, in contrast to the findings of Macpherson (1988) and Macpherson *et al*. (1992), who reported a significant relationship between *in vitro* cariogenic potential and caries experience, it was not possible to discriminate significantly between strains isolated from clinically caries-free children and those with high caries experience.

In her study, Macpherson (1988) commented that

differences in cariogenic potential were observed when strains were tested on separate occasions, and that the observed variability required further investigation.

In Section 3.2.5, differences in the demineralising potential of bovine enamel were proposed as a possible source of variation, and was a contributory factor in the decision to substitute powdered hydroxyapatite in the *in vitro* model (Section 3.4). However, from the studies described in this Chapter, it was apparent that having substituted powdered hydroxyapatite for bovine enamel in the test system, variation on repeated testing was still observed. In the studies just described, strains were incubated in batches of six, with *S. mutans* NCTC 10449 included as a control. Mean calcium release and acid anion production on repeated testing (n=8) of this control strain was 11.73($\pm$2.70) mmol/l and 307($\pm$54) nmol/mg wet weight of bacteria. The coefficients of variation on replicate testing were therefore 23% and 16% for calcium release and acid anion production respectively.

If this model was to be used to determine the cariogenic potential of a large number of bacterial strains on a longitudinal basis, it was considered important to ensure that variability on repeated testing was such that it did not obscure differences in cariogenic potential of different strains. This aspect of the test was investigated further as described in Chapter 7.

CHAPTER 7

VARIATION IN THE *IN VITRO* CARIOGENIC POTENTIAL OF *STREPTOCOCCUS MUTANS*.

7.1.1 Introduction

The studies reported in Chapters 4 and 6, confirmed that by using the model described in Section 3.4, it was possible to demonstrate significant differences in the *in vitro* cariogenic potential of six species of streptococci, and between strains of *S. mutans*. It was, however, apparent from the results obtained with the control strain, *S. mutans* NCTC 10449 (Section 6.2 & 6.3), that unacceptable variation was observed when the cariogenic potential of this organism was assessed on eight different occasions.

It was considered important, that if a large number of strains of *S. mutans* were to be tested on a longitudinal basis, variability on repeated testing must be low in order that relatively small variations in cariogenic potential between strains could be demonstrated.

In determining the cariogenic potential of *S. mutans*, strains isolated from a large number of individuals, there were three main sources of variability, namely between individuals, between strains isolated from one individual, and on repeated testing.

Variability between individuals.

If differences in the cariogenic potential of S. mutans strains were to account for the disparity between salivary counts of S. mutans and caries experience (Section 6.1.4), then it was hoped that the largest source of variation would be between individuals. The larger the range of such differences then the greater would be the chances of demonstrating significant relationships between the cariogenic potential of the strains isolated from different individuals and caries experience, expressed as either prevalence or incremental data.

Variability between strains.

One important factor, not so far considered in the theory that differences in the cariogenic potential of S. mutans strains may account for the discrepancies observed between salivary counts of S. mutans and caries experience (Section 1.18), was potential differences in virulence of different strains harboured by one individual. Also, if the model was to be of clinical value, then it was highly desirable that the variability in cariogenic potential between strains isolated from one individual was much less than the variability between individuals.

Variability across replicates of the same strain.

As already discussed, the less the replicate variability,

the greater would be the ability of the model to discriminate between strains of similar cariogenic potential. For technical reasons, during each experimental run, strains were tested in batches of six. There were therefore two different sources of replicate variability, (i) variability when a strain was tested on two or more separate occasions, and (ii) variability when two or more preparations of the same strain were tested concurrently. Obviously, it was highly desirable that these sources of variability were minimal.

Therefore, to determine the influence of these different sources of variability on the in vitro assay of cariogenic potential, the following study was designed.

7.1.2 Materials and methods

The in vitro cariogenic potential of up to six strains of S. mutans, isolated from each of 24 individuals as described in Section 2.2, was assayed as described in Section 3.4. In addition, during the preparation of the bacterial pellet, when cells were first suspended in potassium chloride, one millilitre was removed and a viable cell count was performed after appropriate serial dilution, inoculation on Columbia blood agar, and incubation.

Individuals with a range of caries experience (DMFS 0-30) were selected for study. A total of 64 strains were

investigated, strains being tested in batches of six, with 24 batches being tested overall. Each batch was designed to examine different aspects of the variability across individuals, strains, batches and replicates, and were as described in Table 7.1. For descriptive purposes individuals are identified by the numbers 1-24, and strains were labelled A-F.

Batches 1-4 tested one strain from each of 12 individuals (strains 1A - 12A), on two different occasions, and were designed to test variability across individuals and between batches.

Batches 5-8 tested the same 12 strains as batches 1-4 and, in addition to providing further information on variability between batches, assessed variability of replicates within the same batch.

In batches 9-12, a second strain from each of the 12 individuals (1B-12B) was tested in combination with strains (1A -12A), thereby providing information on variability across strains isolated from one individual, in addition to providing further information on between-batch variability, of strains (1A - 12A).

In batches 13-16, each batch contained six different strains (A-F), isolated from one individual (subjects 13-

Batch	Strains					
1	1A	2A	3A	4A	5A	6A
2	7A	8A	9A	10A	11A	12A
3	1A	2A	3A	7A	8A	9A
4	4A	5A	6A	10A	11A	12A
5	1A	1A	2A	2A	3A	3A
6	4A	4A	5A	5A	6A	6A
7	7A	7A	8A	8A	9A	9A
8	10A	10A	11A	11A	12A	12A
9	1A	1B	5A	5B	9A	9B
10	2A	2B	6A	6B	10A	10B
11	3A	3B	7A	7B	11A	11B
12	4A	4B	8A	8B	12A	12B
13	13A	13B	13C	13D	13E	13F
14	14A	14B	14C	14D	14E	14F
15	15A	15B	15C	15D	15E	15F
16	16A	16B	16C	16D	16E	16F
17	17A	17A	17A	17A	17A	17A
18	18A	18A	18A	18A	18A	18A
19	19A	19A	19A	19A	19A	19A
20	20A	20A	20A	20A	20A	20A
21	21A	21A	21B	21B	21C	21C
22	22A	22A	22B	22B	22C	22C
23	23A	23A	23B	23B	23C	23C
24	24A	24A	24B	24B	24C	24C

Numbers = Individuals
Letters = Strains

Table 7.1. Experimental batches, designed to examine variability across batches, individuals, strains, and replicates.

16), and were designed to test the variability across strains within the same individual.

Batches 17-20 each tested one strain (A), isolated from individuals (17-20), and were designed to assess variability amongst replicates of the same strain, from one individual, tested simultaneously.

Batches 21-24 contained three different strains (A-C), isolated from individuals (21-24) and replicated within each batch, thereby providing further information on replicate variability and on variability between strains isolated from one person.

Statistical analysis was conducted as described in Section 2.4.2, in conjunction with the Department of Statistics, University of Glasgow.

There were obviously innumerable permutations in which replicates, batches, strains and individuals could have been tested to investigate variability with the in vitro test system. The protocol outlined in Table 7.1, was judged to give adequate appraisal of the factors under investigation. However, statistical analysis of the data was complicated by the fact that confounding occurred in batches 13-24, where a subject appeared in only one batch and was the only subject in that batch. It was therefore

not possible to separate batch and individual variances for batches 13-24.

7.1.3 Results

Mean calcium release, acid anion production and pH after 5 hours' incubation by the strains incubated in batches 1-12, are recorded in Table 7.2. Strains 1A-12A were incubated on four occasions across three batches, whereas strains 1B-12B were incubated on only two occasions in two different batches.

For strains 1A-12A, calcium release ranged from 3.06 (±1.04) mmol/l by strain 11A, to 14.61 (±7.08) mmol/l by strain 6A. Whilst calcium release by some strains was broadly similar on repeated testing, for example, strains 4A (4.97, 6.85 6.44 & 5.73) and 7A (2.94 4.80 5.46 & 3.38), it was apparent for other strains that a range of values was produced when a given strain was incubated on different occasions. In particular, attention is drawn to strain 10A, where calcium release on the four different incubations was, 21.83, 1.49, 3.11 and 3.84 mmol/l and, similarly strain 6A, where calcium release data were 9.00, 11.91, 12.56 and 24.97 mmol/l. Calcium release by strains 1B-12B ranged from 4.58 mmol/l by strain 5B, to 19.02 mmol/l by strain 6B.

For acid production, by strains 1A-12A, minimum and maximum values were again attributable to strains 11A and 6A respectively. However, as was the case with calcium

	Calcium (mmol/l wet wt.)	Acids (nmol/mg wet wt.)	pH
	mean[a] (S.D.)	mean[a] (S.D.)	mean[a] (S.D.)
Batches 1-12			
S. mutans Strain			
1A	12.69 (5.95)	769 (586)	4.35 (0.29)
2A	10.82 (4.39)	448 (190)	4.46 (0.24)
3A	6.88 (3.33)	464 (190)	4.51 (0.28)
4A	6.00 (0.83)	589 (319)	4.49 (0.28)
5A	4.30 (1.58)	458 (233)	4.64 (0.17)
6A	14.61 (7.08)	953 (623)	4.32 (0.23)
7A	4.14 (1.18)	660 (199)	4.64 (0.23)
8A	5.18 (4.06)	406 (396)	4.48 (0.13)
9A	4.47 (2.23)	350 (143)	4.64 (0.10)
10A	7.57 (9.56)	586 (744)	4.64 (0.23)
11A	3.06 (1.04)	257 (99)	4.74 (0.11)
12A	6.70 (2.28)	372 (90)	4.51 (0.30)
	mean[b]	mean[b]	mean[b]
1B	13.64	839	4.50
2B	11.99	761	4.59
3B	9.70	525	4.61
4B	9.98	726	4.56
5B	4.58	320	4.80
6B	19.02	997	4.42
7B	6.40	389	4.72
8B	7.66	405	4.64
9B	4.82	350	4.76
10B	13.57	833	4.55
11B	4.97	453	4.70
12B	6.26	340	4.69

[a] = mean of four determinations
[b] = mean of two determinations
S.D. = Standard Deviation

Table 7.2. Mean calcium release, acid anion production and pH produced by S. mutans strains (1A-12A) and (1B-12B) incubated in batches 1-12.

release, whilst acid production by some strains was broadly similar on repeated testing, with other strains large differences in acid production were observed, examples being strain 10A where 190, 227, 1701 and 225 nmol/mg wet weight bacteria were detected and, similarly, four incubations of strain 6A resulted in values of 1828, 380, 915 and 688 nmol/mg wet weight bacteria.

Acid production by strains 1B-12B was akin to that produced by strains 1A-12A and ranged from 320-997 nmol/mg wet weight bacteria by strains 5B and 6B respectively. However, whilst acid production by some strains was similar on repeated testing (436/469, and 362/338 nmol/mg wet weight bacteria by strains 11B and 9B respectively), in other cases, marked disparity between acid production on repeated testing was observed, e.g. 322/1344, and 327/1195 nmol/mg wet weight bacteria, by strains 10B and 2B respectively.

Minimum and maximum pH values after 5 hours' incubation were again produced by strains 6A and 11A, i.e. 4.32 ($\pm$0.23) and 4.74 ($\pm$0.11) respectively.

Mean calcium release, acid production and pH during the incubations conducted in batches 13-24 are detailed in Table 7.3.

In each of batches 13-16, six different strains isolated from one individual were tested. For each person a range

	Calcium (mmol/l wet wt.)		Acids (nmol/mg wet wt.)		pH	
Batches 13-16						
Strains	mean[a]	(S.D.)	mean[a]	(S.D.)	mean[a]	(S.D.)
13A-13F	8.38	(3.46)	657	(113)	4.54	(0.09)
14A-14F	11.69	(6.66)	662	(220)	4.56	(0.14)
15A-15F	10.07	(2.16)	1081	(402)	4.55	(0.06)
16A-16F	12.56	(4.40)	593	(158)	4.55	(0.08)
Batches 17-20						
Strain	mean[b]	(S.D.)	mean[b]	(S.D.)	mean[b]	(S.D.)
17A	14.59	(1.37)	759	(167)	4.50	(0.05)
18A	9.56	(1.38)	835	(205)	4.58	(0.04)
19A	8.10	(0.98)	696	(202)	4.67	(0.04)
20A	8.04	(3.24)	811	(204)	4.73	(0.03)
Batches 21-24						
	mean[c]		mean[c]		mean[c]	
21A	27.00		1649		4.37	
21B	26.24		1741		4.38	
21C	28.45		1182		4.36	
22A	10.15		836		4.60	
22B	9.73		676		4.61	
22C	8.30		300		4.66	
23A	17.31		1263		4.38	
23B	17.28		876		4.38	
23C	16.04		549		4.44	
24A	6.72		753		4.61	
24B	3.89		540		4.70	
24C	5.19		561		4.71	

[a] = mean of six determinations (i.e. strains A-F x 1)
[b] = mean of six determinations (i.e. strain A x 1)
[c] = mean of two determinations

Table 7.3. Mean calcium release, acid anion production and pH produced by strains incubated in batches 13-24.

of calcium release values was obtained and ranged from 5.60 to 13.47, 4.34 to 17.98, 6.92 to 12.78 and 6.88 to 19.16 mmol/l by strains 13A/13E, 14E/14F, 15F/15B, and, 16B/16A respectively.

A similar comparison of acid production also showed a range of values for different strains isolated from one individual, the range of results obtained being, 521 to 812, 446 to 938, 708 to 1767, and 419 to 798 nmol/mg wet weight bacteria by strains 13D/13E, 14C/14A, 15A/15B, and 16F/16C respectively.

Mean pH for all four individuals was very similar, namely 4.54 ($\pm$0.09) by individual 13, to 4.56 ($\pm$ 0.14) by individual 14.

In batches 17-20, six incubations of one strain, isolated from each of four individuals (17-20), were tested simultaneously. From the results obtained, it was apparent that variation existed when a strain was tested on more than one occasion. However, as shown in Table 7.2, it would appear that the variation observed was less than when strains were tested in different batches (strains 1A-12A).

In batches 21-24, three strains from each of four individuals, were tested in duplicate and relatively large differences were observed between batches. Additionally, the results for all three strains isolated

from one individual were similar (Table 7.3). However, as discussed in Section 7.1.1, in batches 13-24, strains from any one individual never appeared in more than one batch. It was therefore not possible to determine easily if the differences seen between strains isolated from different individuals reflected genuine differences, or if such differences were influenced by variations from batch to batch.

Statistical analysis of data.

As previously outlined (see Section 7.1.1), this experiment was conducted to determine the contribution of different sources of variability (i.e. batch, individual, strain and replicate), to the in vitro assessment of cariogenic potential. Several statistical techniques, namely a Random Effects Model, a Generalised Linear Model and Expected Mean Square analysis, were applied to the data to determine the components of variance.

When the Random Effects Model was applied, batches (1-24) were assigned to six groups (i.e. batches 1-4, 5-8, ..., 21-24), from which it was possible to estimate a combination of variances. The estimates of the sources of variance in this study are reported in Table 7.4, from which the relative contribution of batch, individual, strain and replicate to the overall variance can be determined.

It can be observed that for all three parameters the

	Source			
	$\hat{\sigma}_b$	$\hat{\sigma}_i$	$\hat{\sigma}_s$	$\hat{\sigma}_r$
Calcium (mmol/l)	5.15	1.79	1.54	2.52
Acids (nmol/mg wet weight x10)	24.90	0.00	12.60	24.10
Min pH ($\times 10^{-2}$)	13.40	11.62	4.61	5.66

$\hat{\sigma}_b$ = variability across batches

$\hat{\sigma}_i$ = variability across individuals

$\hat{\sigma}_s$ = variability across strains

$\hat{\sigma}_r$ = variability across replicates

Table 7.4. Variability across batches 1-24 as assessed by a Random Effects Model.

major determinant of variability was that observed across batches. Considering the calcium results, replicate variability was the second largest source of variability, whilst little difference was observed between individual and strain variability.

In the case of acid anion production, again both batch and replicate variability exceeded strain and individual variability, the latter being negligible. Whilst variability across individuals on the basis of the minimum pH data was greater than that observed across strains or replicates, again batch variability predominated.

In defining the Random Effects Model, no account was taken of the fact that in batches 1-12, individual strains appeared in more than one batch, and the data for these batches were therefore reanalysed using a Generalised Linear Model, generated using the GLM command in the Minitab statistical package. However, as can be observed from Table 7.5, such an approach did not alter the findings of the previous analysis, variability across batches again constituting the largest source of variability.

The Generalised Linear Model also permitted both the weight of the bacterial pellet used, and the viable cell count to be assessed as covariates, both individually and in combination. However, neither of these parameters

	Source			
	$\hat{\sigma}_b$	$\hat{\sigma}_i$	$\hat{\sigma}_s$	$\hat{\sigma}_r$
Calcium (mmol/l)	4.55	2.63	0.99	2.63
Acids (nmol/mg wet weight x10)	35.91	15.30	11.26	25.83
Min pH ($\times 10^{-2}$)	18.75	9.48	3.72	8.93

$\hat{\sigma}_b$ = variability across batches

$\hat{\sigma}_i$ = variability across individuals

$\hat{\sigma}_s$ = variability across strains

$\hat{\sigma}_r$ = variability across replicates

Table 7.5. Variability across batches 1-12 as assessed by a Generalised Linear Model.

affected the sources of variance, variance across batches remaining dominant.

The third statistical technique applied to the data, estimated variance from the Expected Mean Square, using the Rummage command in the Minitab statistical package, after normal probability and residual plots had confirmed the data were normally distributed. This analysis also accounts for the appearance of an individual in more than one batch, and examined all batches. As reported in Table 7.6, again the largest source of variance was across batches.

7.1.4 Discussion

From the studies described in Section 6.2 and 6.3, it was apparent that having substituted powdered hydroxyapatite for bovine enamel in the *in vitro* model, there still remained an undetermined degree of variation when strains were tested repeatedly. If the model were to be used to further investigate the potential of caries activity tests based on salivary counts of *S. mutans*, then it was desirable that the variability was greatest across individuals, with much less variability across strains isolated from one individual and minimal replicate variability. The complex design of the current study aimed to assess different sources of variability.

From the results presented in Tables 7.4-7.6, it was

		Source			
		$\hat{\sigma}_b$	$\hat{\sigma}_i$	$\hat{\sigma}_s$	$\hat{\sigma}_r$
Calcium (mmol/l)	(a)	4.52	2.33	0.00	2.63
	(b)	5.33[1]		2.98	1.90
Acids (nmol /mg wet weightx10)	(a)	26.18	10.73	0.00	25.83
	(b)	20.99[1]		20.54	18.45
Min pH ($\times 10^{-2}$)	(a)	18.00	9.20	0.00	8.93
	(b)	35.00[1]		6.90	4.59

$\hat{\sigma}_b$ = variability across batches

$\hat{\sigma}_i$ = variability across individuals

$\hat{\sigma}_s$ = variability across strains

$\hat{\sigma}_r$ = variability across replicates

(a) = batches 1-12
(b) = batches 13-24

[1] due to confounding it was not possible to separate variance due to batch and individual effects in batches 13-24

Table 7.6. Variability across batches 1-12 and 13-24 as determined from the Expected Mean Square.

apparent that variability was greatest across different batches. This finding could theoretically be overcome by including a control strain in each batch, and relating the findings for a strain within a batch to the control. However, the large replicate variability observed precluded such an approach. It was, therefore, not possible to relate in vitro cariogenic potential assessed during this study, to the caries experience of the individuals from whom the strains had been originally isolated.

It was possible that variation in the weight of cells incubated, or in viable cell counts, influenced batch variability. In the course of this study, cells were standardised on the basis of wet weight. The technical difficulties encountered in this approach to standardisation of cells, together with those experienced in accurately determining viable cell counts from samples of small volume, have been discussed previously (Section 4.1.4). Therefore, in the experiments reported in this Chapter, one millilitre of the potassium chloride suspension was removed during preparation of the bacterial pellet and a viable cell count performed. However, as reported in Section 7.1.3, the inclusion of weight of cell used, or viable cell count as covariates in the Generalised Linear Model, either singly or in combination did not affect the variance estimates of any of the parameters used to determine cariogenic potential.

In previous studies (see Sections 3.4.3 and 4.1.3), significant correlations between acid production, calcium release and pH after five hours' incubation were observed, findings which were repeated in the current study, as reported in Table 7.7. In light of the findings in Tables 7.4-7.6, further statistical analyses were performed. The correlations between acid anion production, calcium release and pH were used to identify "outliers", i.e. uncorrelated observations. However, removal of these unusual or inconsistent observations, still resulted in batch and replicate variabilities, which were greater than individual and strain variability. It was therefore apparent that even having removed outlying values (an approach which would be unacceptable in practice), the reproducability of the model was such that it could not be used to reliably investigate a large number of strains of S. mutans on a longitudinal basis.

As described in Chapter 3, the in vitro model employed in this study evolved from a model developed to assess the cariogenic potential of foodstuffs. In the original report of the model (Primrose et al., 1989), the coefficient of variation for calcium release over a 24 hour period when S. mutans NCTC 10449 was incubated with bovine enamel and a sucrose solution was 56%. In the studies of Macpherson (1988), coefficients of variation ranged from 8% to 38%, when six strains of S. mutans were

	Correlation coefficient	p value
Acids vs Calcium	0.80	0.001
Acids vs min pH	- 0.38	0.001
Calcium vs min pH	- 0.52	0.001

Table 7.7. Relationship between acid anion production, calcium release and pH, following incubation of strains of S. mutans as described in Section 7.1.2.

effectively tested on only two occasions, and it was hoped that the substitution of powdered hydroxyapatite for the bovine enamel would overcome such variations. However, the results of the current comprehensive study confirmed that unacceptable variation in the model still existed, with coefficients of variation ranging from 9% to 126%.

In demonstrating differences in cariogenic potential between strains, the smaller the differences which are sought, the more accurate and reproducible is the detection system required. Newbrun (1989), commented on the difficulty in demonstrating innate differences in virulence between pathogens, and this is particularly so between different strains of one species. Previous studies of the cariogenic potential of oral microorganisms have included both animal and <u>in vitro</u> work. Whilst some studies have reported differences in cariogenic potential within species, few have investigated either the number of strains investigated in this study, or reported adequately on the reproducability of the tests. The limitations of some of these investigations are now discussed.

Animal studies have shown differences in cariogenic potential between strains of a given species (Krasse & Carlsson, 1970; Drucker & Green, 1981; Drucker, Shakespeare & Green, 1984). However, in these papers

cariogenicity was often only reported as ordinal data. This is, of course, totally inadequate in assessing more than a few strains, and is insufficiently precise to relate the cariogenic potential of the strains to clinical determinants of caries experience. In the study of Drucker et al. (1984), strains of S. sanguis I were, for example, categorised as being of either nil, low, or moderate cariogenic potential. However, the number of rats infected by individual strains ranged from three to twelve. Additionally, the number of lesions per jaw were reported but no indication of variability was presented. Kohler and Krasse (1990), reported that strains of S. mutans were more cariogenic than strains of S. sobrinus. However, cariogenic potential as assessed in that study using a hamster model, showed coefficients of variation up to 66%. Strains tested across three different experiments also gave considerable variation. Of 11 strains investigated, five were assessed in only two of the three experimental batches, and a further three were tested in only one experimental batch. Another recent study using a rat model, reported S. sobrinus to be more cariogenic than S. mutans, (de Soet et al., 1991). However, in that investigation coefficients of variation of up to 200% were observed when only nine strains of S. mutans were studied. Thus considerable variation is apparent in animal studies of cariogenic potential, as might be expected from the many possible sources of variability in such models. In addition to

standardisation of the initial cell culture, once inoculated, strains must successfully colonize the teeth and overcome the host defence mechanisms of the animal model.

In vitro test systems, potentially offer many advantages over animal models in terms of cost, ease of use and standardisation. Many studies of bacterial metabolism have used cells grown in a chemostat system. This technique provides a long-term, constant, stable and defined environment, which produces a homogeneous population of cells and allows accurate and precise manipulation of culture variables (Tempest, 1978). Continuous culture has been used to investigate the physiology of oral streptococci associated with dental caries (Mayo et al., 1989). However, it is apparent that even when cells have been cultured in a chemostat, and then used in short-term biochemical experiments, considerable variation in acid production by a given strain can be observed. Marsh, Keevil and Ellwood (1984), investigated the relationship of bioenergetic processes to the pathogenic properties of oral bacteria. They reported that acid production by S. mutans Ingbritt ranged from 172 ($\pm$25) to 66 ($\pm$25) nanomoles of acid neutralised / mg cells / minute. Thus over three or four determinations, coefficients of variation ranged from 15% to 38%. Therefore, considerable variation was observed even when cells had been grown in a chemostat and

fermentation experiments were limited to a 15 minute period. Additionally, as discussed previously (Section 4.1.4), it would be impractical to use continuous culture as a means of preparing large numbers of strains for determination of metabolic characteristics.

A further *in vitro* determination of the metabolic characteristics of oral streptococci was reported by De Soet, Toors and de Graff (1989), who investigated acidogenesis by oral streptococci at different pH values. Here, cells were grown using a batch culture technique, standardised on the basis of optical density, with acid production determined by use of a pH stat system. From the data presented, it was not possible to accurately determine the reproducability of their system.

From the foregoing discussion, it is apparent that the difficulties encountered in accurately and reproducibility defining metabolic characteristics of importance in the caries process, are not confined to the study described in this Chapter. These difficulties were confounded by the problems in demonstrating innate differences in strains of one species of bacteria.

It was therefore thought necessary to consider further, potential sources of variation in the *in vitro* model. The decision to incubate the cells for five hours was based on the finding that, after this period, five of the

strains originally investigated by Macpherson (1988), could be significantly differentiated on the basis of acid anion production, calcium release and pH. However, with hindsight, it was apparent that incubation for a shorter interval may have produced an equally significant discrimination between strains. During incubation it became obvious that pH fell most rapidly during the initial incubation period, as can be observed from Figure 4.2. From this graph, differences in the pH of the vials appear more pronounced after 30 minutes or one hours' incubation, than after five hours. It was proposed that if the majority of acid production occurred in the initial period of incubation, then the dissolution of the hydroxyapatite may have been influenced by prolonged exposure to the acid, thus increasing the variability. It was therefore possible that a shorter incubation period may have resulted in a more precise estimation of cariogenic potential, and this concept is discussed further in Chapter 8.

7.1.5 Conclusions

The study described in this Chapter, confirmed that the assessment of cariogenic potential by the <u>in vitro</u> dissolution of powdered hydroxyapatite, was insufficiently precise to permit correlations with the clinical caries experience of the individuals from whom the strains had been isolated originally. The studies described in the following Chapter, were therefore

designed to investigate further potential sources of variation in the *in vitro* model.

CHAPTER 8

AN INVESTIGATION OF FACTORS INFLUENCING THE *IN VITRO* CARIOGENIC POTENTIAL OF *S. MUTANS*.

8.1 The relationship of acid production, calcium release and pH during incubation of *S. mutans* NCTC 10449.

8.1.1 Introduction

Previous studies in this thesis have described the dissolution of powdered hydroxyapatite over a five hour period as a means of determining the *in vitro* cariogenic potential of oral microorganisms. From the study described in Chapter 7, it was apparent that, in its current form, this model was insufficiently precise to permit accurate and reproducible assessment of cariogenic potential of a large number of strains, of a single bacterial species, on a longitudinal basis. However, as discussed in Section 7.1.4, it was possible that incubation for a shorter period would result in a more precise measure of cariogenic potential. Therefore, this study aimed to investigate the relationship between acid production, calcium release and pH, during the five hour incubation period. In light of the media effects reported in Chapter 4, bacterial cells were grown in Todd Hewitt Broth supplied by either Gibco or Oxoid (see Section 4.2). In addition, changes in the viable cell count in the course of the incubation, were investigated.

8.1.2 Materials and methods

The Type strain of S. mutans, NCTC 10449 was selected for study and prepared as described in Section 3.4.2, i.e. the vials contained 40 mg wet weight bacteria, 10 mg powdered hydroxyapatite and 400 µl 5% sucrose solution (w/v). The bacterial cells were tested in six experimental batches, each consisting of six vials. Cells incubated in batches 1-3 were grown in Todd Hewitt Broth supplied by Gibco, whilst those incubated in batches 4-6 were grown in Oxoid Todd Hewitt Broth.

In contrast to previous experiments, where all vials were incubated for five hours, here only one vial in each batch was incubated for the full five hour period, the remaining vials being frozen after 0, 0.5, 1, 2, and 3.5 hours' incubation. Vials were subsequently prepared for determination of acid anion and calcium content (see Sections 2.3 & 2.6).

The protocol for determination of viable cell count differed from that employed in the studies described in Sections 4.2 and 7.1. When the components of the test, (i.e. bacterial pellet, sucrose solution and powdered hydroxyapatite), were combined in the vial and agitated using the vortex mixer to ensure a homogeneous suspension, 10 µl was removed using a sterile Eppendorf pipette and transferred to a plastic bottle containing one millilitre of sterile phosphate buffered saline.

This was then sonicated for fifteen seconds at a setting of 1.5, by means of an ultrasonic sonicator (Heat Systems Ultrasonics, Plainview, USA). The sonicate was then diluted serially, inoculated on Columbia blood agar and incubated for 2-3 days at 37°C, in air and 5% CO_2, when a viable cell count was performed. Samples were removed from the experimental vials for determination of viable cells, at the beginning and end of the incubation period.

Statistical analysis was conducted as described in Section 2.4.2.

8.1.3 Results

Mean acid anion production, calcium release and change in pH during the incubation are reported in Table 8.1 and Figures 8.1-8.3, the results being differentiated on the basis of nutrient media used to prepare the bacterial cells. Values observed for mean acid anion production, calcium release, and pH after five hours' incubation, were 531 ($\pm$17) nmol/mg wet weight bacteria, 0.5 ($\pm$0.01) mmol/mg wet weight bacteria, and 4.04 ($\pm$0.06) by the Gibco prepared cells, and 849 ($\pm$124) nmol/mg wet weight bacteria, 0.91 ($\pm$0.12) mmol/mg wet weight bacteria and 3.89 ($\pm$0.01) by the Oxoid prepared cells respectively.

As reported in previous studies, pH fell most rapidly in the first half hour period of incubation, values dropping from 6.74 ($\pm$0.12) to 4.69 ($\pm$0.05), and 6.76 ($\pm$0.06) to

Time (hours)	Gibco T.H.B.	Oxoid T.H.B.
	Acids (nmol/mg wet weight bacteria)	
	mean[a] (S.D.)	mean[a] (S.D.)
0.0	38 (9)	53 (13)
0.5	165 (42)	235 (48)
1.0	222 (56)	432 (58)
2.0	317 (33)	577 (15)
3.5	407 (148)	841 (49)
5.0	531 (17)	849 (124)
	Calcium (mmol/mg wet weight bacteria)	
	mean[a] (S.D.)	mean[a] (S.D.)
0.0	0.00	0.00
0.5	0.06 (0.02)	0.18 (0.03)
1.0	0.11 (0.08)	0.40 (0.06)
2.0	0.27 (0.04)	0.57 (0.04)
3.5	0.37 (0.14)	0.81 (0.01)
5.0	0.50 (0.01)	0.91 (0.12)
	pH	
	mean[a] (S.D.)	mean[a] (S.D.)
0.0	6.74 (0.12)	6.76 (0.06)
0.5	4.69 (0.05)	4.35 (0.06)
1.0	4.38 (0.19)	4.13 (0.03)
2.0	4.27 (0.06)	4.02 (0.02)
3.5	4.19 (0.10)	3.93 (0.02)
5.0	4.04 (0.06)	3.89 (0.01)
	hydrogen ion concentration mol/l x 10^{-5}	
	mean[a] (S.D.)	mean[a] (S.D.)
0.0	0.02 (0.00)	0.02 (0.00)
0.5	2.05 (0.26)	4.50 (0.62)
1.0	4.46 (1.87)	7.37 (0.52)
2.0	5.41 (0.75)	9.48 (0.46)
3.5	6.62 (1.53)	11.67 (0.61)
5.0	9.10 (1.18)	12.98 (0.71)

[a] = mean of three determinations.

T.H.B. = Todd Hewitt Broth.

S.D. = Standard deviation

Table 8.1. Acid anion production, calcium release, pH and hydrogen ion concentration after varying periods of incubation of S.mutans NCTC 10449.

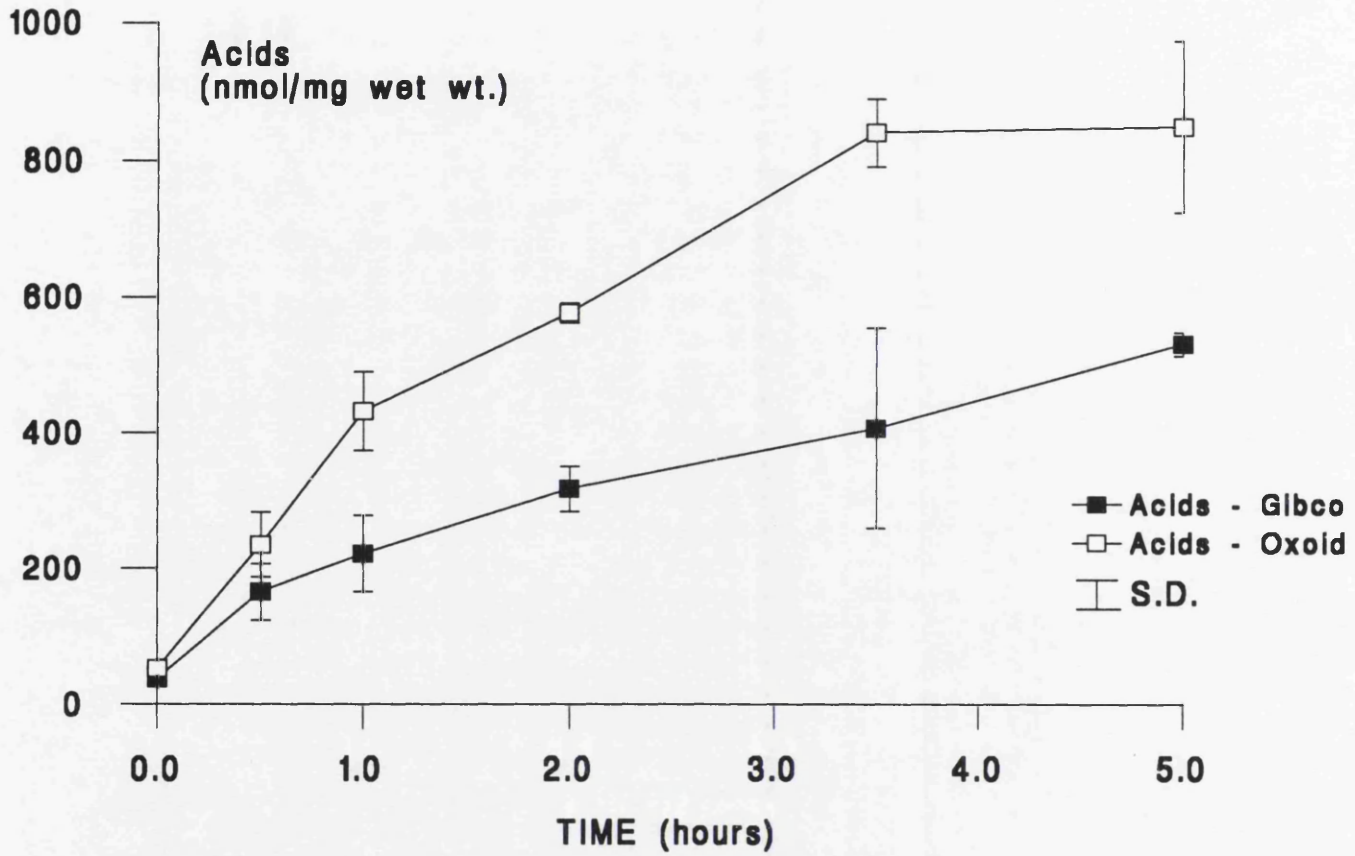


Figure 8.1. Acid anion production during incubation of S. mutans NCTC 10449.

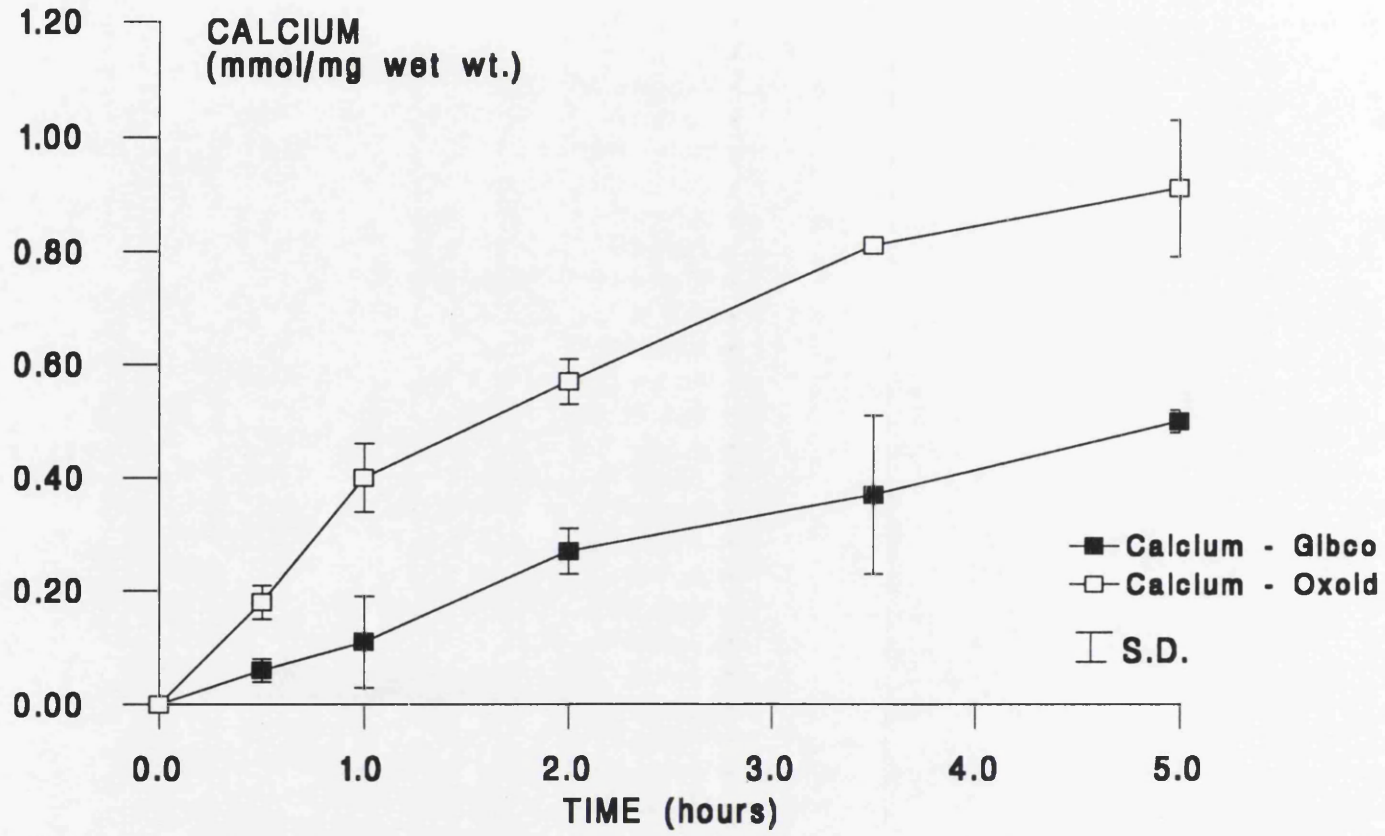


Figure 8.2. Calcium release during incubation of S. mutans NCTC 10449.

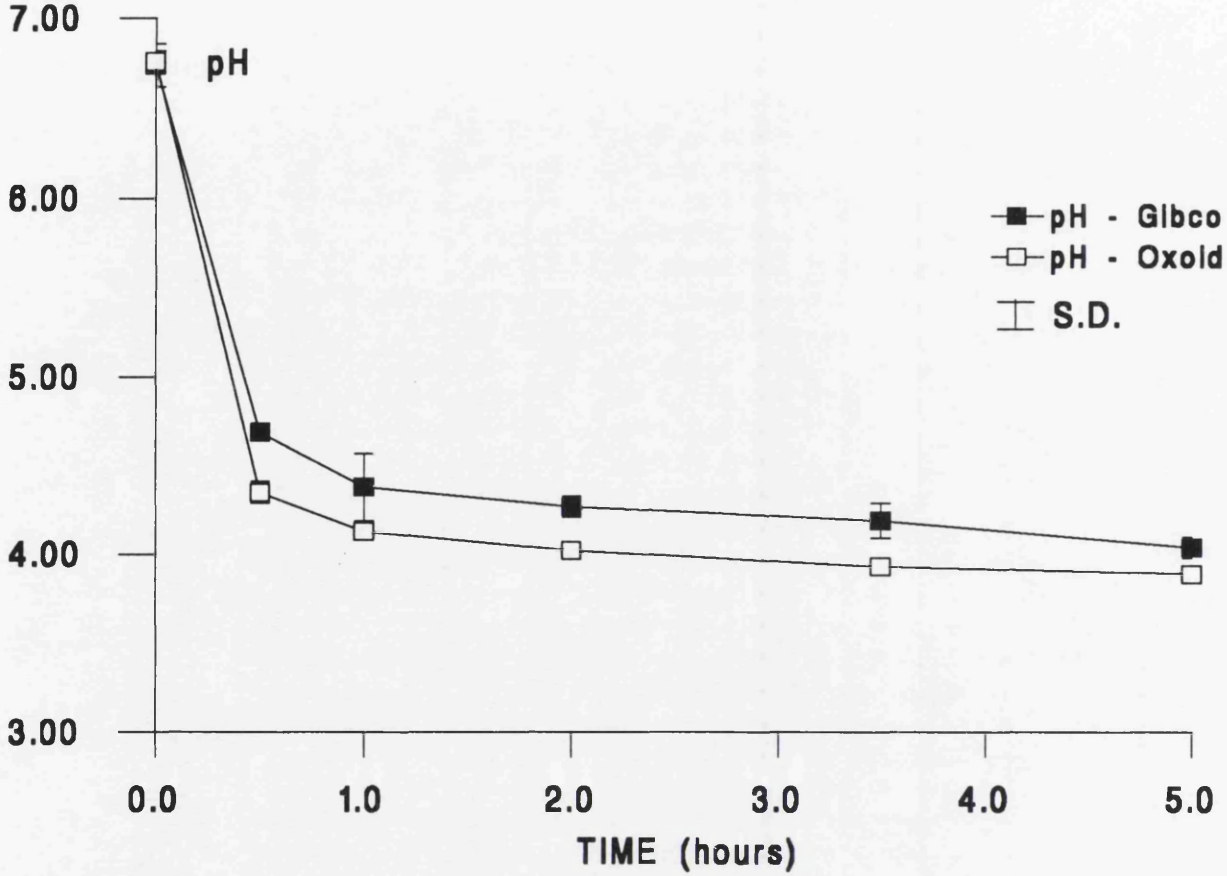


Figure 8.3. Changes in pH during incubation of S. mutans NCTC 10449.

4.35 (±0.06), with the Gibco and Oxoid prepared cells respectively, values representing 75.9% and 83.9% of the total change in pH during the incubation. If, however, the change in hydrogen ion content of the vial supernatant is reported as a concentration (Table 8.1), then it becomes apparent that the increase in hydrogen ions during the first half hour incubation period represents 22.2% and 34.5% of the five hour value.

After 30 minutes' incubation, values for calcium release were 0.06 (±0.02) and 0.18 (±0.03) mmol/mg wet weight of bacteria, which respectively represented 12% and 19.8% of total calcium released over five hours by the Gibco- and Oxoid-prepared cells. A similar comparison of acid anion production in the first half hour of the incubation constituted 25.8% and 22.9% of the total acid production, again by the Gibco and Oxoid cells respectively.

From Figures 8.1 - 8.3, it can be observed that, whilst pH fell most rapidly and the rate of acid production was greatest in the initial part of the incubation, acid production and calcium release continued throughout the incubation. Acidogenesis, by the Oxoid prepared cells, appeared to plateau between three-and-a-half and five hours. However, this finding may be attributable to the variation observed in acid production after five hours' incubation. Similarly, the large variation observed in the acid production by the Gibco-prepared cells after

three-and-a-half hours, further complicated the assessment of the relative rates of acid production in the latter stages of the incubation. The finding of small amounts of acid at time zero, may be accounted for by endogenous metabolism of the bacterial pellet and acid production in the time taken to measure the pH, remove the sample for viable cell count determination, and freeze the vial.

Calcium release continued throughout the incubation and, from Figure 8.3, it is apparent that in the initial stages, both the rate and amount of calcium release was greater with the Oxoid-prepared cells. In agreement with the acid anion determinations, large variability in calcium release was observed after three-and-a-half hours' incubation with the Gibco-prepared cells, and five hours with the Oxoid-prepared cells.

Mean initial viable cell counts ranged from 10.45 ($\pm$0.14) to 10.98 ($\pm$ 0.25) log c.f.u./ml. (Table 8.2). However, in contrast to the findings in Section 4.2.4, no significant differences, as assessed by analysis of variance, were observed in the mean initial cell count either within, or between vials containing cells prepared in either Gibco or Oxoid supplied Todd Hewitt Broth. It is apparent that a reduction in enumerable viable cells occurred in the course of the experiment. However, after five hours' incubation, 9.71 ($\pm$0.57) and 10.03 ($\pm$0.40)

Incubation period (hours)	Mean[a] initial viable cell count (S.D.)	Mean[a] final viable cell count (S.D.)	Percentage reduction viable cells (S.D.)
	Gibco T.H.B.		
0.0	10.92 (0.37)		
0.5	10.53 (0.54)	10.42 (0.94)	1.1 (3.8)
1.0	10.60 (0.48)	9.72 (0.16)	8.2 (3.0)
2.0	10.85 (0.41)	9.69 (0.27)	10.6 (4.1)
3.5	10.98 (0.25)	9.21 (0.76)	16.1 (5.3)
5.0	10.82 (0.51)	9.71 (0.57)	10.2 (5.5)
	Oxoid T.H.B.		
0.0	10.70 (0.22)		
0.5	10.58 (0.40)	10.44 (0.57)	1.3 (4.6)
1.0	10.59 (0.43)	9.92 (0.33)	6.3 (0.8)
2.0	10.67 (0.20)	9.97 (0.27)	6.5 (4.2)
3.5	10.95 (0.26)	10.06 (0.50)	8.0 (6.0)
5.0	10.45 (0.14)	10.03 (0.40)	4.8 (6.2)

Viable cell counts are expressed as log c.f.u./ml.

[a] = mean of three determinations.

S.D.= Standard Deviation.

Table 8.2. Changes in viable cell counts of S. mutans NCTC 10449 during determination of in vitro cariogenic potential.

log c.f.u./ml viable cells were still detectable in the Gibco- and Oxoid-prepared cells respectively.

8.1.4 Discussion

This study was conducted to provide further information on changes in the determinants of cariogenic potential and viable cell counts in the course of the five hour incubation period. The decision to incubate the cells for this time was based on the findings of the study reported in Section 3.4. However, in retrospect, it was thought possible that a more precise determination of cariogenic potential might have been achieved by incubating the cells for a shorter time period. The results of the current study showed that, whilst the pH fell most rapidly in the initial incubation period when hydrogen ion production was reported as a concentration, close correlations with acid anion production and calcium release were observed. If the majority of acid production had occurred in the initial period of the incubation, it was possible that dissolution of the hydroxyapatite, over the five hour period, could have been related to the presence of the acid rather than been directly influenced by the metabolism of the bacterial cells. However, from Table 8.1 and Figures 8.1 and 8.2, it was apparent that, whilst the rate of acid production and calcium release was higher in the earlier stages of incubation (with the exception of calcium release by the Gibco-prepared cells), both acid production and calcium

release continued throughout the five hour incubation period. This indicated that *in vitro* cariogenic potential was influenced by bacterial metabolism throughout the experiment. As stated previously, the variability observed in acid anion production and calcium release in the latter stages of the experiment, complicate the assessment of the contribution of bacterial metabolism to cariogenic potential in the final 90 minutes of incubation.

The continuance of acid production is, of course, influenced by a number of factors, namely the availability of substrate, the build-up of metabolic products, and continued viability of the cells, factors which will now be considered in greater detail.

Due to the concentration of sucrose used, it is unlikely that sucrose acted as a limiting factor in this experiment, as was discussed in Section 6.3.4.

The aciduric properties of *S. mutans* are believed to contribute to the cariogenic potential of this species (Hamada & Slade, 1980), and differences in the cariogenic potential of the strains may be reflected in their ability to metabolise carbohydrate at depressed pH levels. Harper and Loesche (1983), and De Soet *et al.* (1989) suggested that species of mutans streptococci differed in their patterns of metabolic stimulation and

inhibition at different pH levels. It is likely that such properties are influenced by the length of time the cells have been subjected to a strongly acidic environment. It could therefore be argued that the longer the period of incubation, the greater are the chances of demonstrating differences in the cariogenic potential of the strains.

Viable cell counts were conducted in the studies described in Sections 4.2 and 7.1, and the limitations of the techniques used on those occasions have been discussed. In the current study, after the addition of the powdered hydroxyapatite, samples were removed for determination of viable cell counts at the beginning, and at the end of the respective incubation periods. The presence of the hydroxyapatite complicated the determination of viable cell counts and, prior to serial dilution, the sample was sonicated to encourage disaggregation of bacterial cells, both from each other and from the hydroxyapatite. As can be observed from Table 8.2, the experimental vials contained a very high bacterial cell count.

It was apparent that a reduction in viable cell count occurred in the course of the experiment (Table 8.2), a result which confirmed that nett proliferation of the bacterial cells did not occur during the incubation. This was, of course, an expected finding in view of the constituents of the experimental vial. However, it was

not possible to determine to what extent the reduction in cell numbers reflected, (a) cell death during the experiment, (b) increased adsorption to the hydroxyapatite, or (c) coaggregation of the cells. Nonetheless, surprisingly high viable cell counts (in the order of 10^{10} cells/ml), were still detectable at the end of the incubation period.

In conclusion, the results of the current study indicate that, contrary to initial expectations, a lesser period of incubation would not necessarily have resulted in a more precise measure of cariogenic potential, and would not necessarily have eliminated the variation observed in the study described in Chapter 7. It may, in fact, be argued that incubation for a shorter period would tend to reduce the contribution of the aciduric properties of the strains to the determination of cariogenic potential. Furthermore, reducing the incubation period may have reduced the discriminating power of the model, as a minimum period is required to demonstrate differences between strains. It will, however, be remembered that in the study described in Section 3.4, the significance of the differences between the strains was less after 24 hours, than after five hours' incubation.

8.2 The effect of the components of the *in vitro* model on cariogenic potential.

8.2.1 Introduction

The following experiment was designed to investigate the effect of the interaction of the constituents of the experimental vials on the determinants of *in vitro* cariogenic potential.

8.2.2 Material and methods

As outlined in Table 8.3, six experimental vials containing various combinations of bacterial cells, powdered hydroxyapatite, sucrose solution and sterile distilled water were incubated for a five hour period. As *S. mutans* NCTC 10449 had been used as a control strain throughout this thesis, and the majority of experiments had been conducted using the Gibco-supplied Todd Hewitt Broth, this strain grown in the Gibco broth was prepared for incubation, as described in Section 3.4. Thereafter, vials were centrifuged and both acid anion and calcium content determined as described in Section 2.3 and 2.6. In addition, viable cell counts were conducted as described in Section 8.1.2.

The contents of vials A-F were as follows:

(1) Vial A, was incubated as a control, and contained 40 mg wet weight bacterial pellet, 10 mg powdered hydroxyapatite and 400 µl 5% sucrose solution (w/v).

Vial	400 µl 5% sucrose solution	40 mg bacterial cells	10 mg powdered hydroxyapatite
A	+	+	+
B	-[a]	+	-
C	-[a]	+	+
D	+	+	-
E	+[b]	+	+
F	+	-	+

- = absent

+ = present

[a] = 400 µl sterile distilled water (i.e. no sucrose).

[b] = 400 µl 5% sucrose (w/v) in 135 mM KCl. (i.e. sucrose suspended in 135 mM KCl soln.)

Table 8.3. Contents of experimental vials incubated as described in Section 8.2.

(2) Vial B contained 40 mg wet weight bacterial pellet and 400 µl sterile distilled water.

(3) Vial C contained 40 mg wet weight bacterial pellet, 400 µl sterile distilled water and 10 mg powdered hydroxyapatite.

(4) Vial D contained 40 mg wet weight bacterial pellet and 400 µl 5% sucrose solution (w/v).

(5) Vial E contained 40 mg bacterial pellet, 400 µl 5% sucrose solution (w/v) in 135 mM KCl, and 10 mg powdered hydroxyapatite.

(6) Vial F contained 400 µl 5% sucrose solution (w/v) and 10 mg powdered hydroxyapatite.

Vials were incubated in batches of six (A-F), on three separate occasions.

Statistical analysis was conducted as described in Section 2.4.2.

8.2.3 Results

As reported in Table 8.4, after five hours' incubation maximal acid production occurred in vial E, 490 (± 248) nmol/mg wet weight bacteria, but was not significantly different from that observed in vial A, 459 (±44) nmol/mg wet weight bacteria, as assessed by analysis of variance. An intermediate value of 194 (±66) nmol/mg wet weight bacteria of acid production was observed in vial D, with minimal acid anions detected in vials B and C. As

Acid anions nmol/mg wet weight bacteria

	mean[a]	(S.D.)
A	459	(44)
B	47	(24)
C	44	(33)
D	194	(66)
E	490	(248)
F	0	

Calcium mmol/l

Vial	mean[a]	(S.D.)
A	7.66	(1.59)
B	0.00	
C	0.00	
D	0.00	
E	8.20	(1.64)
F	0.00	

pH

	mean[a]	(S.D.)
A	4.37	(0.05)
B	6.09	(0.04)
C	6.55	(0.02)
D	4.11	(0.05)
E	4.58	(0.07)
F	6.78	(0.04)

[a] = mean of three determinations.

S.D. = Standard Deviation.

Table 8.4. Acid anion production, calcium release and pH after five hours' incubation of the experimental vials described in Section 8.2.2.

expected, no acids were detected in vial F.

In addition to quantitative differences in acid anion production, differences were observed in the proportion of specific acid anions comprising total acid production, as reported in Table 8.5. Lactate was the predominant acid anion in vials A and E, with values of 89.4 (±3.1)% and 92.8 (±3.0)% respectively. In vial D, whilst lactate again predominated, accounting for 69.1 (±2.4)%, formate, pyruvate and succinate accounted for 22.3 (±1.0)% of acid production in this case. In those vials lacking sucrose, namely B and C, acetate constituted 35.6 (±6.8)% and 32.2 (±19.3)%, and lactate 43.2 (±24.2)% and 40.9 (±16.6)% of acid production respectively.

Calcium was detected only in vials A and E with values of 7.66 (±1.59) and 8.20 (±1.64) mmol/l vial supernatant respectively.

In considering the pH of the vial contents after five hours incubation, values of 4.37 (±0.05) and 4.58 (±0.07) were observed with vials A and E respectively. However the lowest pH value was detected in vial D, in which only moderate amounts of acid anions were detected. In accordance with acid production, minimal depression in pH occurred during the incubation of vials B and C, whilst the pH of vial F remained constant throughout the incubation.

Vial	Proportion of specific acid anions comprising total acid anion production.					
	Lactate		Acetate		Others	
	mean[a]	(S.D.)	mean[a]	(S.D.)	mean[a]	(S.D.)
A	89.4	(3.1)	3.5	(1.0)	7.0	(3.1)
B	43.2	(24.2)	35.6	(6.8)	21.2	(27.0)
C	40.9	(16.6)	32.2	(19.3)	26.9	(36.0)
D	69.1	(2.4)	8.6	(2.9)	22.3	(1.0)
E	92.8	(3.0)	4.1	(1.0)	3.1	(2.7)
F	-		-		-	

[a] = mean of three determinations.

S.D. = Standard Deviation.

Table 8.5. Proportion of specific acid anions comprising total acid production in vials (A-F), incubated as described in Section 8.2.

Mean viable cell counts ranged from 9.93 (±0.10) log c.f.u./ml in vial D, to 10.35 (±0.02) log c.f.u./ml in vial B, no viable cells being detected in vial F. Over the five hour incubation period, a reduction in mean viable cell counts was detected, mean percentage reductions ranging from 3.91 (±0.73)% in vial C, to 17.03 (±2.64)% in vial D, as illustrated in Figure 8.4.

8.2.4 Discussion

This experiment was performed to gain further information on the interaction of *in vitro* test components and to identify possible sources of variation in the model identified in studies described in Chapter 7.

Vial A contained all three elements of the model as originally described (Section 3.4), and was included to act as a control. Vial B was designed to determine the contribution of the bacterial cells to acid anion and calcium concentration, in the absence of sucrose or powdered hydroxyapatite. Vial C contained bacterial cells, hydroxyapatite, and sterile distilled water, and was intended to investigate the effect of the presence of the hydroxyapatite on endogenous metabolism. Vial D contained only bacterial cells and sucrose solution, and aimed to investigate bacterial metabolism in the absence of the powdered hydroxyapatite.

The inclusion of vial E was to study the effects of the potassium chloride supplemented sucrose solution, on the

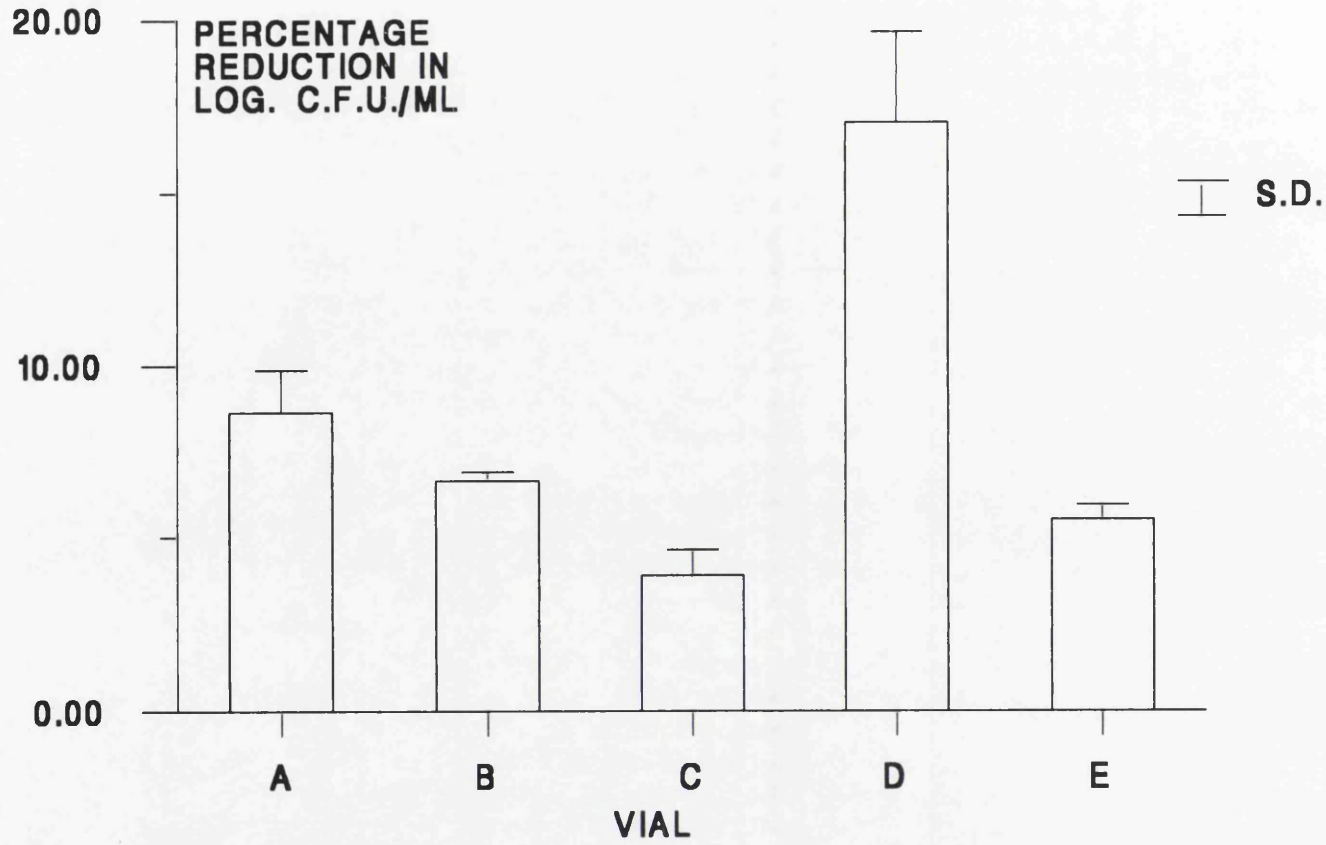


Figure 8.4. Mean percentage reduction in log. c.f.u./ml during incubation of vials A-E as described in Section 8.2.

determinants of in vitro cariogenic potential. Marsh et al. (1984), have shown that by washing and incubating cells in potassium chloride, the resultant high intracellular potassium means that energy does not have to be expended to maintain the normally low intracellular sodium ion and high potassium ion concentration, a process that will de-energise the membrane. Thus, the potassium chloride treated cells have an increased transport capacity and therefore increased ability to produce acids. As described in Section 3.4, during the preparation of the bacterial cell pellet, cultures were washed in 135 mM potassium chloride. However, the sucrose solution used in the in vitro model was prepared using sterile distilled water, therefore, in vial E, the sucrose was dissolved in 135 mM potassium chloride. Finally, vial F contained only the sucrose solution and powdered hydroxyapatite, and was designed to confirm that changes in calcium content of the vial were dependant on the presence of the bacteria.

As reported in Table 8.4, mean acid production in vial E was not significantly different from that observed in vial A. The failure to demonstrate significantly increased acid production in vial E was attributed to the fact that all cells were washed in potassium chloride during preparation of the bacterial pellet, and it is probable that even in vial A, the extracelluar fluid of the bacterial pellet was rich in potassium ions. The

greater variability in acid determination observed in vial E, may be attributable to the chloride ions in the supernatant which, during isotachophoresis, increased the zone length of the leading electrolyte, and thus increased the time taken for acid anions to reach equilibrium (see Section 2.3 & 2.4). The greater variability in vial E may, therefore, be due to technical reasons rather than to an inherent microbial effect.

The production of significantly less acid in Vial D than in vials A and E, can be attributed to the effect of hydroxyapatite on acid production.

In vivo oral microorganisms mainly exist, either attached to mucosal or tooth surfaces, or to each other in the form of dental plaque. However, many of the previous studies of oral streptococcal metabolism have been conducted in liquid culture, and there is relatively little information on the effect of solids on bacterial metabolism. Zobell (1943), in a study of marine bacteria, found that the rates of multiplication and respiration were higher in bacteria exposed to glass surfaces than those free in suspension. These findings were supported by Stotzky and Rem (1966), who demonstrated that adsorption to clay minerals enhanced the respiratory rate of a wide spectrum of bacteria. Berry and Henry (1977), reported enhanced metabolic activity of S. mutans strains while adsorbed to hydroxyapatite, as compared to hydroxyapatite-free

control suspensions. These findings support the results obtained in vials A, D and E in the current study.

Whilst several explanations have been offered to explain the beneficial effects of solid surfaces on bacterial activity, the precise mechanism of the effect remains unclear. Zobell (1943) stated that the adsorbent surface stimulated increased bacterial multiplication and oxygen uptake, whilst Stotzky and Rem (1966) postulated that ion-exchange mechanisms exhibited by the adsorbent, may play a role in the stimulation of metabolism by certain soil bacteria. In the current experiment, bacterial cells were present in high concentration (in the order of 10^{10} cells per ml), and vortex mixing at the start of the experiment (and prior to pH determinations) ensured that a close physical relationship between the cells and the hydroxyapatite was maintained during the incubation. However, the proportion of the cells actively adsorbed to the hydroxyapatite remains uncertain.

The lower pH in vial D, compared to that observed in vials A and E, is probably due to the absence of the buffering effect of phosphate released from the hydroxyapatite. Therefore it is possible that in addition to the physical effect of the hydroxyapatite on metabolism discussed above, phosphate acted as a buffer, thereby creating a less acidic environment and leading to the increased acid production observed in vials A and E. However, Berry and Henry (1977) reported that the

addition of buffering systems to compensate (or nullify) any buffering action of the hydroxyapatite, failed to offset the enhanced acid production in the presence of hydroxyapatite. They proposed that other aspects of metabolic enhancement contributed to the increased acid production seen in the presence of the hydroxyapatite.

In vials B and C, the small amounts of acids detected were attributed to endogenous metabolism of the cells. However, in contrast to the exogenous metabolism, this was unaffected by the presence of hydroxyapatite.

In the original in vitro model (see Section 6.3.3), lactate represented the major acid anion produced, findings which were again confirmed in vials A and E. However, whilst lactate predominated in vial D, the minor acids constituted over one fifth of the total acid production in the absence of hydroxyapatite.
In contrast to these findings, acid production in the absence of an external carbohydrate source yielded similar quantities of lactate and acetate. From Table 8.5, the greatest variability in the proportion of acid anions was observed in vials B and C. This finding is likely to reflect the technical difficulty in detecting acid anions by isotachophoresis when present in trace amounts, as discussed in Section 2.4.4.

Calcium was detected only in vials A and E and, whilst

mean calcium release was higher in the presence of the potassium chloride supplemented sucrose solution, the difference was not significant. It is therefore inferred that the dissolution of sucrose in potassium chloride prior to incubation would not have significantly affected the results of the previous studies of in vitro cariogenic potential and may, as already discussed, actually have increased the variability.

Whilst acid anions were detected in vial C, any resultant calcium release was not observed using the method described in Section 2.6. It was therefore judged that endogenous metabolism did not contribute to calcium release. The failure to identify calcium in vials B and D, confirmed that detectable calcium levels were dependant on the presence of the hydroxyapatite. The findings in vial F confirmed that detectable levels of calcium release were dependant on the presence of the bacterial pellet.

Whilst, in general, the pH of the vial contents agreed with observed changes in acid anion content after five hours' incubation, the lowest pH was detected in vial D which contained less acids than either vials A or E. This finding supports the theory that phosphate released during the dissolution of the hydroxyapatite exerted a buffering effect on vial contents as discussed above.
The changes in viable cell counts observed in this study

were similar to those described in Section 8.1. As can be observed from Figure 8.4, the greatest reduction in viable cell counts occurred in vial D. Whilst the difficulties encountered in conducting viable cell counts discussed previously are equally valid, the findings in vial D may have resulted from a greater cell death due to the lower pH observed in the absence of the hydroxyapatite buffering effect.

To conclude therefore, whilst preliminary experiments not reported in this thesis had confirmed, for example, that powdered hydroxyapatite was insoluble in a sterile sucrose solution, factors such as the contribution of endogenous metabolism, intra-or extra- cellular calcium stores etc., to the determinants of <u>in vitro</u> cariogenic potential, were judged not to have been fully investigated. Whilst this study was again conducted using only one bacterial strain, no parameters were identified which would satisfactorily account for the variability observed between either batches or replicates in the study described in Chapter 7.

This study has, however, highlighted that bacterial metabolism can be significantly influenced by the presence of solid surfaces, and studies using inert surfaces are required to further our understanding of this potentially very important, but little understood phenomena.

CHAPTER 9

9.1 CONCLUDING DISCUSSION

Whilst many studies have investigated the microorganisms associated with dental caries, most have either been of an epidemiological nature, or have examined the role of only a limited number of bacterial strains in either animal or *in vitro* models. As a result of the work by Macpherson (1988) and Macpherson *et al*. (1992), it was thought possible that the investigation of potential differences in the cariogenicity of strains harboured by individual patients, could improve the accuracy of microbiological-based caries-activity tests to assess the risk status in individuals.

A test system designed to investigate the *in vitro* cariogenic potential of individual isolates of bacteria was adapted from the model described by Macpherson (1988), as described in Chapter 3. In developing the model, the objective was to design an experimental system which could be used to investigate a large number of strains on a longitudinal basis. It was also considered desirable that the test could be adapted for routine use in a microbiology laboratory. The substitution of powdered hydroxyapatite for bovine enamel was seen as contributing to these aims by reducing the incubation period required, in addition to removing potential differences in the solubility of the bovine enamel.

The model thus devised, was used to investigate six species of streptococci of differing cariogenic potential, as described in Section 4.1. That study demonstrated that the results obtained with the in vitro test system correlated with previous investigations of these species in animal models. However, the study reported in Chapter 5, failed to establish significant relationships between the in vitro determinants of the cariogenic potential of pooled dental plaque samples collected under field conditions, and clinical caries activity. The reasons why such a relationship may not have been observed were discussed in detail in Section 5.1.4.

The experiments conducted in Chapter 6, returned to the principal aim of the thesis, namely to investigate the in vitro cariogenic potential of S. mutans strains isolated from children participating in a clinical dentifrice trial. If the sensitivity and specificity of caries-activity tests based on salivary counts of S. mutans were to be improved by taking into account differences in virulence of the strains harboured by individuals, a number of prerequisites required satisfaction. Firstly, strains of S. mutans must differ in their cariogenic potential, and the virulence of the strains harboured by an individual patient should be less than observed across individuals. Secondly, a range of virulences would have to be demonstrated in order that association with caries

activity could be demonstrated. Finally, it must be possible to measure the virulence determinants in a standardised reproducible fashion.

The study described in Chapter 6 showed that whilst it was possible to demonstrate differences in the cariogenic potential of selected strains of S. mutans, a significant relationship with the caries experience of the individuals from whom the strains had been originally isolated could not be observed. As stated in Chapter 7, variability observed on repeated testing precluded the investigation of any meaningful association with clinical disease activity. Whilst potential sources of variation were investigated as described in Chapter 8, no parameters were identified which could account satisfactorily for the variation observed. As a result of these studies, the model was judged to be insufficiently precise to discriminate reliably between strains of an individual bacterial species.

In the studies described in this thesis, it was presumed that when a pellet of bacterial cells was prepared in a standardised fashion, the acidogenic potential of the cells would be constant. This assumes that it is possible to control the metabolic activities of individual bacterial isolates with a sufficient degree of precision to consistently demonstrate any differences between them. The difficulties encountered in preparing

a standardised bacterial pellet, and the advantages and disadvantages of continuous culture systems, were discussed in Section 4.1.4.

It may, however, be speculated that whilst it is possible to achieve a pellet of bacterial cells of constant mass and viable cell numbers, such manipulations may influence genetic factors controlling acid production in a non-standardised fashion. Thus, factors governing acid production, such as the presence of constitutive or inducible enzymes (Edwardsson 1986), or of the cell transport mechanisms (Marsh et al. 1984), may not have been controlled sufficiently by the experimental procedures employed.

It was possible to discriminate significantly between the different species of streptococci, probably because their acidogenic potential was sufficiently different. However, the unexplained variation inherent in the model, prohibited reliable discrimination between individual strains of S. mutans, with the notable exceptions of the selected strains described in Chapter 3, and Section 6.3.

The influence of external parameters on acidogenesis of the cells was highlighted further by the finding that acid production could be influenced significantly by growing the cells in Todd Hewitt Broth supplied by two manufacturers.

The *in vitro* model measured principally the acidogenic and, to a lesser extent, aciduric properties of the organisms tested. Previous authors have investigated the acidogenic and aciduric properties of oral microorganisms by means of a pH stat (Marsh *et al*. 1982; De Soet *et al*., 1989), thus enabling a greater degree of environmental pH control. In a preliminary study not reported in this thesis, the acidogenic and aciduric properties of five *S. mutans* strains were investigated using a pH stat. It was possible to demonstrate that acidogenesis of the strains was influenced by environmental pH, the values investigated ranging from pH 7.0 to 3.5. However, acid production by individual strains did not differ at the various pH levels tested.

There are, of course, other determinants of the cariogenic potential of oral microorganisms, (Section 1.5), e.g. intra and extracellular polysaccharide production. It is possible that consideration of these factors may shed further light on the virulence characteristics of individual *S. mutans* strains. However, the technical difficulties encountered in the current study of standardisation and reproducability are equally applicable to investigations of these parameters.

Finally, as discussed Chapter 1, the aetiology of dental caries is multifactorial in nature. Whilst numerous studies have reported significant relationships between

levels of S. mutans and caries experience, it is unlikely that the caries-risk of an individual patient could be predicted reliably, by consideration of only one causative factor. In a recent report, Disney et al. (1992) concluded that the inclusion of microbiologically-based caries-activity tests in a multiple predictive model, contributed little to model accuracy and probably did not justify the expense incurred in their use. That study, in agreement with most recent investigations of caries-risk, concluded that clinical predictors such as past disease experience, proved most useful as determinants of future disease activity. This is perhaps not surprising considering the multifactorial nature of the disease process. It does, unfortunately, result in the at-risk individual being identified only after experiencing the disease.

In conclusion, the theory that differences in the cariogenic potential of oral microorganisms may help explain the limited usefulness of current caries-activity tests remains attractive. Experience gained in the course of the work described in this thesis would suggest that such differences are unlikely to be elucidated by studying physiological or metabolic characteristics. As suggested by Russell (1991), the development of genetic fingerprinting may provide the answers to questions such as, "How many types of S.mutans does an individual carry?"; "How stable is the oral population?"; "How is

S.mutans transmitted?"; "Are particular clonal lines of S. mutans more cariogenic than others?" or "Do high-risk individuals carry particular types?".

It is therefore possible to speculate that, as molecular biological techniques are developed, it may become possible to identify more accurately differences between strains of S. mutans, and that such differences may correlate with the caries experience of the individuals from whom they were isolated.

APPENDIX A

Constituents and preparation of bacteriological media.

All media were prepared with an aseptic technique, and plates were poured in a clean air laminar flow hood, with the exception of the Columbia Blood Agar, which was poured in an automatic plate pourer.

Solid media.

Each plate contained 15-20 ml agar and, when cool, was stored at 4°C. Prior to use, representative control plates were selected at random from each batch and were incubated for 24 hours to check contamination. In the case of selective media, quality control was confirmed by the routine inoculation and incubation of a standard range of type strains.

Liquid media.

Sterile broths were stored at room temperature, and prior to use, sterility was confirmed visually and at intervals by culture of a broth sample.

ANAEROBIC BLOOD BROTH.

Supplier:- Gibco, Paisley, Scotland.

Stated constituents:-

	per litre
Pancreatic casein digest	12.000 g
Beef extract	4.000 g
Yeast extract	5.000 g
Sodium carbonate	0.106 g
Sodium bicarbonate	0.900 g
Dithiothereitol	0.100 g
L cysteine	1.000 g
Liver digest	3.000 g
Glucose	5.000 g
Sodium chloride	3.840 g
Vitamin K	0.005 g
Haemin (Bovine)	0.005 g

Preparation.

1 Weigh out 32 g of medium per litre of broth required.

2 Add to distilled water and mix thoroughly until dissolved.

3 Aliquot in 20 ml volumes.

4 Autoclave at 121°C for 15 minutes.

5 Store at room temperature prior to use.

Final pH = 7.4 +/- 0.2

BRAIN HEART INFUSION AGAR.

Supplier:- Difco, East Molesley, England.

Stated constituents:-	per litre
Calf brains, (infusion from),	200.0 g
Beef heart, (infusion from),	250.0 g
Proteose peptone	10.0 g
Bacto dextrose	2.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g
Bacto agar	15.0 g

(May be adjusted to allow for variation in raw materials.)

Preparation.

1 Rehydrate in distilled water, 52 g of medium per litre of agar required.

2 Dissolve in a Koch steamer.

3 Cool to 50°C and adjust to required pH by incremental addition of glacial acetic acid (BDH Chemicals Ltd, Poole England). pH was measured using a 220 pH meter (Ciba Corning Diagnostics Ltd., Suffolk, England) and general purpose combination electrode (Kent Industrial Measurements Ltd., Gloucestershire, England.)

4 Autoclave at 121°C for 15 minutes.

5 Allow to cool to 50°C and pour plates.

BRAIN HEART INFUSION BROTH.

Supplier:- Difco, East Molesley, England.

Stated constituents:-	per litre
Calf brains, (infusion from),	200.0 g
Beef heart, (infusion from),	250.0 g
Proteose peptone	10.0 g
Dextrose	2.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g

May be adjusted to allow for variation in raw materials.

Preparation.

1 Weigh out 37 g of medium per litre of broth required.

2 Add to appropriate quantity of distilled water and dissolve in Koch steamer (Laboratory Thermal Equipment Ltd., Oldham, England).

3 Aliquot in appropriate volumes.

4 Autoclave at 121 °C for 15 minutes.
Final pH = 7.4 +/- 0.2

COLUMBIA BLOOD AGAR.

Supplier:- Gibco, Paisley, Scotland.

Stated constituents:-	per litre
Peptone 140 (Pancreatic digest of casein)	13.0 g
Peptone 100 (Peptic digest of animal tissue)	6.0 g
Yeast extract	3.0 g
Beef extract	3.0 g
Starch	1.0 g
Sodium chloride	5.0 g
Agar	13.0 g

7.5% DEFIBRINATED HORSE BLOOD

Supplier:- Gibco, Paisley, Scotland.

VITAMIN K AND HAEMIN SOLUTION

Supplier:- Gibco, Paisley, Scotland.

Preparation.

1 Weigh out 44 g of medium per litre of agar required.

2 Add to distilled water and dissolve in Koch steamer, mixing thoroughly at frequent intervals.

3 Autoclave at 121 °C for 15 minutes.

4 Cool to 50 °C and add 7.5% sterile defibrinated horse blood (75 ml blood per litre of agar), and 1% sterile vitamin K and haemin solution to give a final concentration of 5 mg/l haemin and 0.5 mg/l vitamin K.

5 Mix by swirling to avoid bubbles and pour plates. Final pH 7.3

MITIS SALIVARIUS BACITRACIN AGAR.

(Gold, Jordan and van Houte, 1973)

Supplier:- Difco, East Molesley, England.

Stated constituents:-	per litre
Bacto tryptose	10.0000 g
Proteose peptone No 3, Difco	5.0000 g
Proteose peptone, Difco	5.0000 g
Bacto dextrose	1.0000 g
Bacto saccharose	50.0000 g
Dipotassium phosphate	4.0000 g
Trypan blue	0.0750 g
Bacto crystal violet	0.0008 g
Bacto agar	15.0000 g

(May be adjusted to meet performance specifications.)

Preparation.

1 Weigh out 90 g of medium and 20 g of sucrose (BDH Chemicals Ltd, Poole, England) per litre of agar required.

2 Dissolve in appropriate quantity of distilled water in Koch steamer mixing thoroughly at frequent intervals.

3 Autoclave at 121°C for 15 minutes.

4 Cool to 50°C and add 1 ml of 0.1% Chapman Tellurite and 1 ml of 20 units/ml Bacitracin (Sigma Chemical Co. Poole, England) per 100 ml of agar.

5 Mix by swirling to avoid bubbles and pour plates. Final pH 7.0

ROGOSA AGAR. (Rogosa et al, 1951)

Supplier:- Difco, East Molesley, England.

Stated constituents:-	per litre
Bacto tryptone	10.00 g
Yeast extract	5.00 g
Bacto dextrose	10.00 g
Bacto arabinose	5.00 g
Bacto saccharose	5.00 g
Sodium acetate	15.00 g
Ammonium citrate	2.00 g
Monopotassium phosphate	6.00 g
Magnesium sulphate	0.57 g
Manganese sulphate	0.12 g
Ferrous sulphate	0.03 g
Sorbitan monooleate	1.00 g
Bacto agar	15.00 g

Preparation.

1 Weigh out 75 g of medium per litre of agar required.

2 Add to distilled water and dissolve in Koch steamer mixing thoroughly at frequent intervals.

3 Remove from steamer and add 1.32 ml of glacial acetic acid (BDH Chemicals Ltd, Poole, England), per litre of agar.

4 Mix well and boil for a further 2-3 minutes.

5 Cool to 50 °C, mix by swirling to avoid bubbles and pour plates.

Final pH 5.4

Preparation of carbohydrate media for fermentation tests.

Basal Media.

THIOGLYCOLLATE MEDIUM (without dextran or indicator).

Supplier:- Difco, East Molesley, England.

Stated constituents	per litre
Bacto yeast extract	5.00 g
Bacto casitone	15.00 g
L-cysteine, Difco	0.25 g
Sodium chloride	2.50 g
Thioglycollate acid	0.30 ml
Bacto agar	0.75 g

Preparation

1 Weigh out 24 g of thioglycollate medium per litre of media required.

2 Add to appropriate volume of distilled water and dissolve in a Koch steamer, agitating frequently.

3 Aliquot in 90 ml volumes.

4 Autoclave at 121°C for 15 minutes.

Preparation of glucose, mannitol, melibiose, raffinose, and sorbitol.

5 Dissolve 1 g of carbohydrate in 10 ml of distilled water.

6 Filter sterilise using 0.2 μm filter (Sartorius, Gottingen, Germany).

7 Add the 10 ml of carbohydrate medium to the 90 ml of warm basal medium. This mixture is then dispensed in the microtitre plates as required (see Section 2.2.3).

TODD HEWITT BROTH (I).

Supplier:- Gibco, Paisley, Scotland.

Stated constituents:-	per litre
Beef heart infusion	3.1 g
Peptone 180	20.0 g
Dextrose	2.0 g
Sodium chloride	2.0 g
Sodium phosphate dibasic	0.4 g
Sodium carbonate	2.5 g

Values represent typical formulations. Each production lot is adjusted to compensate for variables in and/or availability of raw materials.

Preparation.

1 Weigh out 30 g of medium per litre of broth required.

2 Add to appropriate volume of distilled water and dissolve in a Koch steamer agitating frequently.

3 Aliquot into 20 ml volumes.

4 Autoclave at 121 °C for 15 minutes.

5 Store at room temperature.

Final pH 7.8 +/- 0.2 at 25 °C.

TODD HEWITT BROTH (II)

Supplier:- Oxoid, Basingstoke, England.

Stated constituents:-	per litre
Infusion from 450g fat-free minced beef	10.0 g
Tryptone	20.0 g
Dextrose	2.0 g
Sodium bicarbonate	2.0 g
Sodium chloride	2.0 g
Disodium phosphate, anhydrous	0.4 g

Preparation.

1 Weigh out 36.4 g per litre of broth required.

2 Add to appropriate volume of distilled water and dissolve in a Koch steamer agitating frequently.

3 Aliquot in 20 ml volumes.

4 Autoclave at 121 °C for 15 minutes.

5 Store at room temperature.

Final pH approximately 7.8

APPENDIX B

Preparation of standard acid solution for isotachophoresis.

A standard acid solution containing formate, pyruvate, phosphate, lactate, succinate, acetate, and propionate (BDH Chemicals Ltd., England) was prepared using the following formula,

$$\frac{MW \times 5 \times 100}{SG \times \% \text{ Purity}}$$

where

MW = Molecular Weight

SG = Specific Gravity

% Purity = Manufacturers declared purity of reagent.

As the exact volume determined by the above formula was difficult to achieve in preparing the standard acid solution due, for example, to the viscosity of the pyruvic acid, the concentration of the individual acid anions in the standard acid solution was not always exactly 5mM. Therefore, as reported in Table 2.2, due allowance was made in calculating the constants c and m.

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