THE DEVELOPMENT AND APPLICATION OF AN IN VITRO TEST FOR THE DETERMINATION OF THE CARIOGENIC POTENTIAL OF FOOD.

by

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SUMMARY

Over the past decade, the general public have become more aware of the influence they can have on their general and dental health by modifying their diet. With this in mind health care professionals need to be aware of the relevant information concerning different foodstuffs, so that reliable information can be relayed to their patients.

The work detailed in this thesis was carried out to develop a rapid, inexpensive and simple *in vitro* test, to determine the cariogenic potential of foods. This was necessary to maximise the available resources by indicating which foods would merit testing in more exhaustive methods.

The model which was modified was that of Geddes *et al*, 1984, an *in vitro*, 5 day recycling microfermentation system, which used human plaque and human enamel. The modifications which were carried out were those recommended by the Scientific Consensus Conference on Methods for the Assessment of the Cariogenic Potential of Foods (1986). This involved incubating a slab of bovine tooth, which had the enamel surface abraded and was coated with an acid resistant varnish leaving an enamel window, with a homogenous slurry of *Streptococcus mutans* type c, grown to early stationary phase in a chemically defined medium, as an artificial plaque and substrate. The bacteria/substrate mixture was changed daily, and the incubation was carried out for 2 incubations (with the same tooth slab throughout).

Having made the required modifications to the Geddes *et al* model, the developed system was tested using 5% (w/v) sucrose, 5% (w/v) sorbitol and water, to ensure that it could

discriminate these substrates. The parameters of pH, organic acid anions formation (using isotachophoresis), calcium release and lesion formation (using microradiography and microdensitometry) were examined. The model could adequately distinguish the positive sucrose control from the negative sorbitol control and water substrates.

Solutions of other sugars were then tested to ensure that the model was able to discrimate more finely. Five per cent solutions of lactose, galactose and xylitol were tested in the model and the various parameters measured as before. The model found the cariogenic potential of the sugar solutions to be (from highest cariogenic potential to lowest) : Sucrose > Lactose = Galactose > Sorbitol > Xylitol. These results were consistent with those found by other workers using different systems.

Having tested various sugar solutions, foods which had already been used by other workers, in other systems were tested, to ensure that the developed system could handle foodstuffs. Milk chocolate, mint-flavoured sweets and raw peanuts were chosen, and processed in such a way that their state resembled that found after mastication. The test was carried out as before and the cariogenic potential obtained (again ranked from highest to lowest): Milk Chocolate = Mint-flavoured Sweets > Sucrose > Peanuts > Sorbitol. These results were in agreement with those obtained by other workers.

Unknown foods could then be tested in the system, and Bombay Mix (a curried mixture of lentils, chickpeas, peanuts and soya crisps) and Tropical Treats (dried bananas,

pineapples, raisins, peanuts and coconuts) were tested, as these has been recommended as 'safe snacks' from a low fat, high fibre consideration. The results obtained were: Sucrose = Tropical Treats > Bombay Mix > Sorbitol.

In this model it could be possible that foods, such as Bombay Mix, containing high amounts of starch, would show a much lower cariogenic potential than they would in vivo, due to the absence of salivary amylase. In a further experiment, salivary amylase was added at a physiological concentration, to see if the results obtained were significantly different from those obtained in the absence of salivary amylase. Addition of salivary amylase made no difference to the results of sucrose, sorbitol and Tropical Treats. The results were significantly higher for starch and Bombay Mix and were significantly lower for peanuts. However, in vivo, 24 hour contact with salivary amylase is unrealistic.

These foods were then tested in a human plaque pH test using the sampling method, to see how the in vitro method compared with an in vivo. The results showed that the in vivo situation gave results closer to the in vitro model without the addition of salivary amylase.

In summary, this work has developed a quick, easy and inexpensive primary screening test for the determination of the cariogenic potential of foods. Foods of unknowm cariogenic potential can be screened in this model, and an assessment of their suitability to be tested in the more time-consuming and expensive models obtained, thus making best use of the available resources.

SYNOPSIS

There is an increasing awareness on the part of the general public about health care. People are becoming more concerned about the ways they can become and stay healthy. In order to assist the public, professionals in all aspects of health care need to be acquainted with the relevant facts to enable them to give reliable and helpful information. In the area of dental health, the professional needs not only to provide conservative treatment, but also to offer preventive care. It is in the area of preventive dentistry that cariogenicity testing falls. Obviously, in order for the dentist to give the public information concerning the cariogenic potential of various foodstuffs, the foodstuffs need to be tested and their cariogenic potential assessed.

Over the past 30 years, many different tests have been described. However, in 1985 a conference was held in San Antonio, Texas, which many of the major workers in the field of cariogenicity testing attended. At this meeting, the various methods currently employed to assess the cariogenicity of foodstuffs were evaluated and the ways tests should be used and developed in the future discussed. The conference recommended that foodstuffs be assessed as to their cariogenic potential using either the human plaque pH model or the animal caries model, both of which have problems, for example with either obtaining volunteers and/or, being time-consuming and expensive. Obviously a screening test which was quick, inexpensive and easily performed would be useful to determine which foodstuffs

would merit being tested in these more extensive systems. The delegates at San Antonio recommended that the *in vitro* de-/re-mineralisation models should be developed further to provide additional tests for cariogenicity testing.

The aim of this work was to develop an *in vitro* test for primary screening of foods to allow a large number of foodstuffs to be quickly and cheaply screened prior to testing in the more extensive models and therefore optimise the available resources. The test uses enamel, cariogenic bacteria and exogenous carbohydrate.

The San Antonio conference recommended the use of *Streptococcus mutans*, type c, grown to early stationary phase, abraded bovine or human enamel and sucrose and sorbitol as positive and negative controls respectively. The model chosen for development was that of Geddes, *et al* (1984), which used an *in vitro* recycling, demineralisation micro-fermentation system with human plaque and human enamel. This system monitored the pH, the production of acid anions using isotachophoresis, total calcium released using a colorimetric assay and the enamel was assessed using the Knoop hardness technique.

In order to develop this model, modifications were made in the following order: plaque experiments were carried out to determine the optimal conditions for the bacterial system; a convenient bacterial system was developed; the techniques for preparation and analysis of the enamel blocks were obtained and the appropriateness of sucrose and sorbitol as positive and negative controls respectively was ensured.

Initially, a series of experiments was performed using the Geddes et al (1984) model to determine the concentration of sucrose required for optimal acid production and the amount of organic acids produced from such an incubation. Starved human plaque and human tooth slabs were incubated with 0, 5, 10 and 20% (w/v) (0, 146 , 292 and 584 mM) sucrose solutions for 5 days, replacing the plaque and substrate daily to provide pH cycling. The pH and acid anion production were measured at various intervals throughout the experiment. The results showed that maximal acid production occurred at sucrose solutions in the 5 - 10% (w/v) range and that the acid production was of the order of 0.3 mmol/mg wet weight plaque. The acid anion concentration was a sum of the formic, pyruvic, lactic, succinic, acetic and propionic acids produced in the incubation (determined by isotachophoresis). The next stage of development was to obtain a bacterial monoculture system which mimicked these results as closely as possible.

A defined bacterial growth medium for Streptococcus mutans was made up and a growth curve for the bacterium obtained to determine the length of incubation required for the organism to be in early stationary phase. This was found to be between 18 and 22 hours. Experiments were then carried out to determine if pre-incubations in media containing different sugar substrates would be required for the bacteria to induce the necessary enzymes to utilise sugar: this was found to be unnecessary. The next stage was to obtain the concentration of bacteria which would produce a system as close as possible to human plaque in

the parameters of rate of pH fall and acid anion production. This was determined by varying the weight : volume ratio of bacteria to substrate. Five percent (w/v) sucrose was used as a substrate and the ratio of 3µl substrate / mg wet weight bacteria gave the best conditions.

The next stage was development of enamel preparation and analysis techniques. Fresh bovine teeth were cleaned and were surface abraded by ~100µm (measured using a modified digital micrometer). They were then varnished on all sides using a proprietary nail varnish leaving an enamel window of measured area (approximately 2mm X 2mm). These slabs were then incubated in the system for the necessary length of time. Following the incubation, the slabs were washed and sectioned to ~250µm using a microtome, then hand-ground to ~100µm before being radiographed and having microdensitometric profiles obtained. The slabs were incubated in the system for different numbers of daily cycles to find the optimal incubation length (to allow lesion formation but not so much enamel breakdown that tissue processing was impossible) - this was found to be 2 days.

The next step was to ensure sucrose and sorbitol were adequate as positive and negative controls respectively. Water, 5% (w/v) sucrose and sorbitol (146 and 274 mM respectively) solutions were used as substrates in a series of experiments and the 0 and 24h results of pH, acid anions and calcium release for sucrose vs sorbitol and sorbitol vs water compared. The results from the sucrose were

significantly different ($p<0.001$ using a Student's t-test, and at the 95% Confidence Interval using Scheffé's test) from both the sorbitol and the water and there were no significant differences between the sorbitol and water substrates. These experiments verified the use of sucrose and sorbitol as positive and negative controls.

To check that the system was able to distinguish sugars of different cariogenic potential, different sugars of known cariogenic potential were tested. Five percent (w/v) solutions of lactose, galactose and xylitol (146 , 277 and 328 mM respectively) were chosen as a range of cariogenic potentials. These sugars were tested and the results obtained ranked by comparing the pH, acid anion and calcium concentration results. The results obtained were placed in rank order (from highest cariogenic potential to lowest): Sucrose > Lactose = Galactose > Sorbitol > Xylitol (> = significantly different using a Student's t-test at $p<0.001$, and at the 95% Confidence Interval using Scheffé's test). These results are consistent to those expected using these sugars, thus showing that this test is capable of differentiating sugars of different cariogenic potentials.

Foodstuffs which had already been tested by other workers in other models were then investigated to see how the models compared, as well as to handle food preparation in the system. Milk chocolate, mint-flavoured boiled sweets and peanuts were chosen as test foodstuffs and prepared to 5% (w/v) mixtures with double-distilled , de-ionised water. The experiment was carried out as before

and the results showed that Milk Chocolate = Mint-flavoured Sweets > Sucrose > Peanuts > Sorbitol(statistical analysis as above). These results confirmed that this test is capable of differentiating foodstuffs of different cariogenic potentials and showed similar results to those found by other workers using different model systems.

Foodstuffs of unknown cariogenic potential were then tested in this system. The Scottish Health Education Group had asked that Bombay Mix (a curried mixture of lentils, chickpeas, peanuts and soya crisps) and Tropical Treats (dried bananas, pineapples, raisins, peanuts and coconuts) could be tested, with a view to the investigation of the cariogenic potential of snacks low in animal fat and high in fibre. These foodstuffs were tested in the model and the following results obtained: Sucrose = Tropical Treats > Bombay Mix > Sorbitol(the results being obtained as above and using the same statistical analysis).

In this model it could be possible that foodstuffs, such as Bombay Mix, containing high amounts of starch would show a much lower cariogenic potential than they would <u>in vivo</u> due to the absence of salivary amylase. In a further experiment, salivary amylase was added to see if the results obtained were significantly different from those obtained in the absence of amylase. Human salivary amylase was added to the substrate mixture in the ratio of 0.38 g/l, which is quoted as the concentration of the enzyme in 'normal' human, whole saliva. The test was repeated using 5% (w/v) mixtures of sucrose, sorbitol, starch, peanuts, Bombay Mix and Tropical Treats as substrates. Addition of

amylase made no significant differences to the results of sucrose, sorbitol and Tropical Treats. Results were significantly higher for starch and Bombay Mix and were significantly lower for peanuts. However, *in vivo* 24 hour contact with salivary amylase is not realistic.

These foodstuffs were then tested in an *in vivo* plaque pH model using the sampling method to compare the results obtained *in vivo* with those obtained *in vitro* without salivary amylase and those obtained *in vitro* with salivary amylase. The results showed that the *in vivo* situation was closer to the *in vitro* model without the salivary amylase, presumably due to the unrepresentative length of exposure to the enzyme in the *in vitro* model.

In summary, this work has developed a quick, easy and inexpensive primary screening test for the determination of the cariogenic potential of foods. Foodstuffs of unknown cariogenic potential can be screened in this model and an assessment of their suitability to be tested in the more time-consuming and expensive models obtained, thus making optimal use of the available resources.

CHAPTER 1

INTRODUCTION

1.1. What is Caries?

In 1940, Robert Stephan described caries thus: 'Caries is a dissolution of the calcium salts from enamel and dentin by acids, formed in the mouth by fermentation by bacteria of carbohydrates lodged on tooth surfaces. This process is followed by disintegration of the organic matrix by proteolytic action of the same or other bacteria.' (Stephan, 1940). A more recent definition of caries is given by Newbrun, (1978), who said the following: 'Dental caries is a multifactorial disease involving three principal factors: host (particularly saliva and teeth), microflora, and their substrate (i.e. diet). A fourth dimension, time or frequency, when considering diet, plays an integral role.' With regard to this second definition particularly, it must be borne in mind that without any one of these three principal factors, caries will not occur, and despite the subject of this thesis being the diet, the contributions of all the factors must be remembered by the reader when considering the results. Fig.1.1. shows a schematic representation of this multifactorial model, and the different components of this model will be dealt with in depth in 1.5..

1.2. History Of Dental Caries.

A primitive pattern of dental caries, where the prevalence is low and the site of attack is generally at or near the enamel-cement junction, has been found in both animal and ancient human remains. This pattern has been

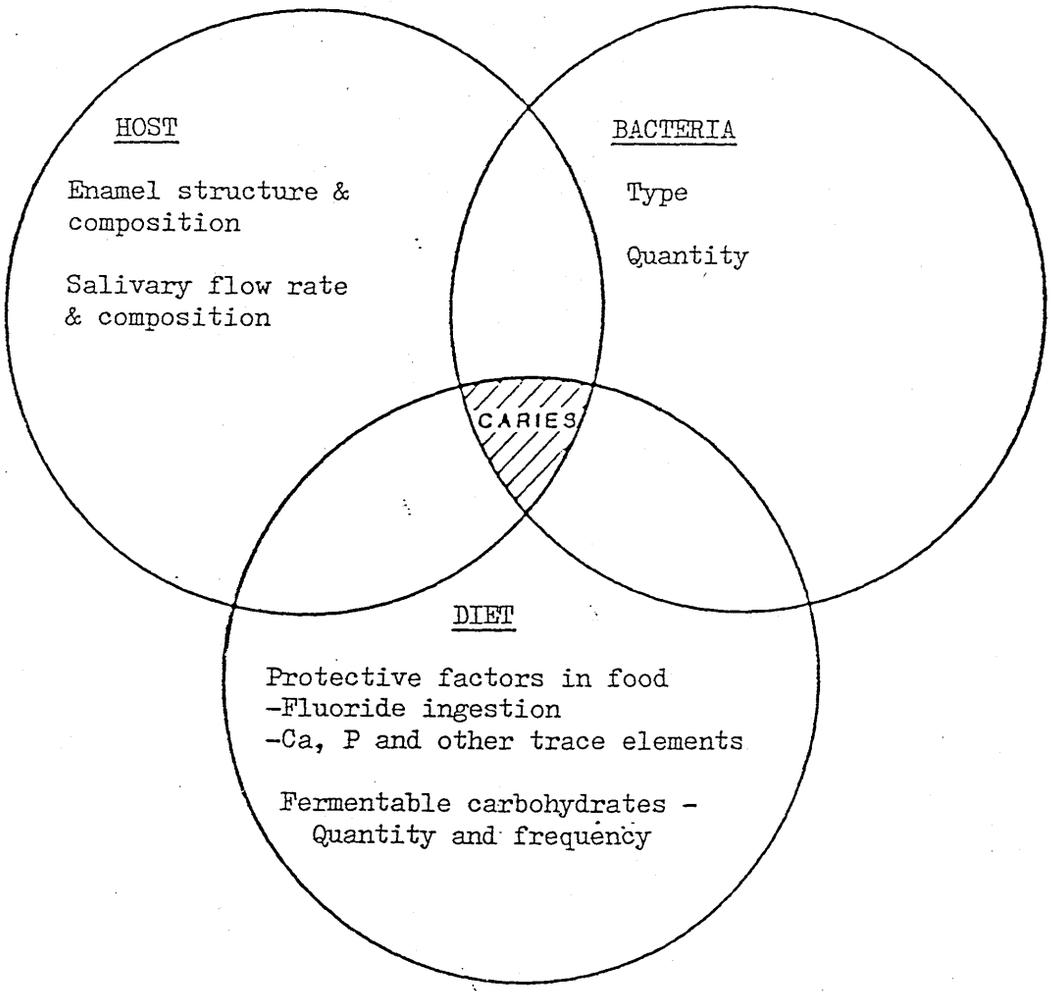

Fig.1.1. Multifactorial Model of Dental Caries.

found in remains from the Iron Age to the end of the medieval period, a span of around 2000 years, and did not appear to change until the 17th century, when trade with the New World sugar industry became established (Moore and Corbett, 1971, 1973 and 1975). Thereafter, the caries pattern changed to the modern one, of high caries prevalence and the location of attack at contact areas and in occlusal fissures (Hardwick, 1960). As a result of this change, the dietary component of dental caries was observed by Pierre Fauchard, and others, who noticed that it was their rich patients who exhibited the most decay and associated this with their ability to buy sugar, which was then an expensive commodity.

1.3. Prevalence Of Caries and Its Link With Sugar Consumption.

Dental caries is the most widespread of all the diseases in the developed world. Todd and Whitworth (1974), found that 44% of Scottish adults over the age of 16 were edentulous and similarly, Todd and Walker (1980), found 29% of the adult population of England and Wales had no natural teeth, and those that had some/all their own natural dentition, showed evidence of caries. Anderson, et al, (1982) demonstrated that there has been a reduction in the caries experience in some groups of English schoolchildren over a period of 5 and 10 years. They cite personal communications that repórt similar reductions in Scotland and other areas in England to those they looked at. They suggest various reasons for this

change although cannot conclude that any one factor is the major contributor. Factors such as change in dietary habits, particularly the change in sugar consumption, fluoridation of water supplies and natural cyclical variations have been suggested. There could also be a contribution from the fact that better preventive dental care is operative throughout the schools service and elsewhere. Diesendorf (1986), reviewed the literature on the reduction in caries prevalence in Australia, Denmark, Holland, New Zealand, Norway, Sweden, United Kingdom, and the United States. These studies had been carried out in areas where the water was unfluoridated, so the preventive effect of water fluoridation could not be the answer. He speculated as to the reason for the decline in caries experience, and though like Anderson, et al, could not give any conclusive answers, postulated that the reductions could be due to topical fluoride, school dental health programmes, a lower sugar intake, the widespread use of antibiotics, the increase in total fluoride intake from the environment and various cyclical causes which had not been investigated. The author of this report felt that although fluoride had an important role in improving caries incidence, more fundamental research was required into the effect of diet and the role of individual immunity from caries. In a recent paper, Downer (1991), summarised the possible reasons for the decline in dental caries, and suggested that further reductions in caries experience could be seen if water fluoridation was more widely practised.

There have been various epidemiological studies carried out in an attempt to clarify the link between caries formation and sugar consumption. The workers concerned have used a number of different approaches to this effect, which are detailed below. Marthaler (1978), looked at the link between sugar consumption and caries production in terms of the annual sugar consumption per capita compared to the DMFT for 11 - 12 year old children from 19 countries. He found there to be a close association, although one criticism could be that due to the sample selected, there was experimental bias on behalf of the link, this being one of the most 'at risk' groups for caries experience. There have been numerous before and after studies, which have looked at groups or populations of peoples both before and after an increase in sugar consumption. Bang and Kristoffersen (1972) showed the results in caries experience of Eskimos of increased trading with the outside world between the initial examinations in 1955 -1957 and the second examinations in 1965. The examinations were all carried out by the same examiner, so the criticism cannot be levelled that different criteria were being employed. They found that between 1957 and 1965 the mean dmft in 1.5 - 5 year old children went from 4.1 to 10.3, and the mean DMFT in 6 - 12 year olds went from 0.7 - 2.4, with the contribution in calorie intake of protein falling from 33 - 15% and from carbohydrate rising from 26 - 45% over the same time span. Holloway, et al., (1963), when studying the population of Tristan da Cunha, a remote Atlantic island, found that pre-

1940, the diet of the inhabitants was very low in sugar and caries experience was also very low. In subsequent examinations in 1952 and 1962, both the caries experience and the sugar consumption had increased markedly, especially in the younger population, the sugar consumption increase being due to a store starting to sell imported sugar and sugar-containing produce. During the time of the Second World War and beyond, sugar consumption was restricted by rationing, and various attempts have been made to correlate this reduction in sugar intake with the caries experience of the population. Workers have reported that in 1945 the sugar consumption per capita was 30 kg/year compared to 52 kg/year in 1961. Many countries have found that reductions in caries prevalence were seen during the period of sugar reduction.(Takeuchi, 1961)

There have been studies on groups of people eating low sugar diets, and two main groups can be discerned. The Hopewood House study, and patients with Hereditary Fructose Intolerence. Hopewood House is an orphanage in New South Wales (Harris, 1963), which has about 80 children who live in the institution, under close supervision until about 12 years of age. The diet provided for the children is very strict following the recommendations of the founder of the institution. The diet virtually excludes sugar and white flour and dental examinations were carried out annually between 1947 and 1962. The examinations found that there was a very low prevalence of caries, compared to similar children attending state schools. After the age of 12 the rate of caries prevalence increased as close supervision

ended, thus intimating that the special dietary regimen did not confer any protection from caries in subsequent years.

Hereditary Fructose Intolerence is a rare disease, which, as the name suggests confers an inability on the sufferer to ingest foods containing fructose. This is a congenital metabolic error which can only be treated by the patient replacing their fructose and sucrose with starchy foods. Marthaler (1967) and Newbrun (1978) report that patients in the age range 6 - 8 years had a very low caries incidence, and in the group over half were caries free.

Another group studied was that of people who for various reasons have a high level of sugar consumption. These include children on long-term syrup containing medication, children given sugar comforters over long periods of time and patients with phenylketonuria. Roberts and Roberts (1979) studied the effect of syrup medicines taken over at least 6 months on the caries experience of 44 children. They found that the mean dmfs of the children taking the syrup medicine was much higher than that of the control children not having the sugar containing medication. It has been seen (Winter et al., 1966 and 1971) that the addition of sugar to an infant's bottle, particularly at night is significantly related to the incidence of rampant caries in the children studied. Winter, et al, (1974) studied the effects of a high carbohydrate intake on patients with phenylketonuria (PKU). PKU is a rare inherited condition which is a result of the patient being deficient in the enzyme phenylalanine hydroxylase. This condition needs to be detected in the

first few weeks of life, as, unless the phenylalanine content of the diet is strictly limited severe mental damage occurs. The diet is modified to have a high carbohydrate content (around 70% of the total carbohydrate ingested), and be low in phenylalanine. Between meal snacks are encouraged to ensure an adequate calorie intake, but chocolate and toffee are prohibited because of their high phenylalanine content. Children studied in this group were found to have the same caries experience as children not experiencing this condition.

1.4. Different Theories Of Caries.

There are generally held to be three main theories of dental caries: i) the chemoparasitic theory,

ii) the proteolytic theory, and

iii) the chelation theory.

The chemoparasitic theory of dental caries was first postulated in 1883 by W.D.Miller, and has since been refined and is the generally accepted theory. As a result of this the following discussion will dwell mostly on this theory before going on to consider the alternative hypotheses. The chemoparasitic theory at its most basic level postulates that caries results when acid produced by oral bacteria in response to a fermentable carbohydrate challenge, reacts with the inorganic component of the tooth enamel and causes its breakdown into calcium and phosphates. The next major input to this theory came from Stephan (1940), who found that pH of plaque fell upon contact with fermentable carbohydrate in a characteristic

pattern - the 'Stephan Curve'. His results confirmed that the pH in plaque following exposure to fermentable carbohydrates was sufficient to cause enamel dissolution in saliva. This characteristic pattern of pH fall and rise has been documented by many other workers, and has given rise to the term 'critical pH' (Guggenheim and Mühlemann, 1978) ie. the pH above which enamel would not dissolve. This value has since been found not to be absolute, depending on such things as the natural resistance of the enamel and the composition of the saliva, but has a range of 5.5 to 6.5. Featherstone, et al, (1979), further refined this theory and postulated a detailed mechanism for caries. They suggested an eleven point mechanism which may be summarised as follows:

i) Plaque bacteria produce organic acids in response to a carbohydrate challenge.

ii) The surface of the enamel is partly protected by an adsorbed substance, perhaps the acquired pellicle.

iii) Unionised organic acids diffuse into the enamel down their concentration gradient.

iv) As the organic acids diffuse some dissociate into their ionic form until at any point an equilibrium is reached.

v) The dissociated acids attack the apatite lattice at points of high solubility and solution equilibria are formed.

vi) Calcium dihydrogen phosphate and calcium hydrogen phosphate as well as calcium salts compounds form and diffuse out of the enamel along their concentration gradients.

vii) As this more soluble fraction of enamel dissolves, the Ca^{2+} and PO_4^{3-} concentrations increase until calcium hydrogen phosphate may precipitate in the translucent zone (a detailed description of the microscopic and microradiographic appearance of the carious lesion is given in Chapter 4). This can then dissolve as more organic acid diffuses in and hence the lesion body is formed.

viii) An intact surface zone is formed as Ca^{2+} and PO_4^{3-} from the subsurface dissolution, repairs the surface crystals.

ix) The rate of inward diffusion of organic acids is greater than the outward diffusion of calcium and phosphate containing species and therefore an equilibrium is achieved at which the rate of diffusion of mineral out of the enamel is equal to the rate of repair.

x) Remineralisation occurs if the calcium and/or phosphate gradients are reversed and the concentration of organic acids is low enough.

xi) HF may be transported as an unionised form and may aid in the removal of H^+ and the F^- may thus be incorporated into the lattice structure.

The proteolytic theory was first hypothesised by Gottlieb in 1944. The theory suggests that the first stage in caries formation is that of proteolytic degradation of the organic component of enamel by the oral bacteria. The chelation theory is a modification of this, in that the products of the proteolysis act as chelators to complex with the calcium formed by the breakdown of the inorganic matrix. This modification was first postulated by Schatz,

et al, (1959). Jenkins (1978), suggests that although these processes may occur in vivo, they would not produce enough demineralisation to result in caries.

1.5. Mechanism Of Caries.

In 1.1., we mentioned the multifactorial nature of dental caries, and in this section we will look in more detail at each of the components.

1.5.1. Host.

There are two factors to this host component, the tooth itself and saliva. The factors important in determining how susceptible a tooth may be to a cariogenic challenge depend on both the individual tooth, its fluorapatite content, degree of maturation, any enamel defects and its location in the mouth, and the association of the different teeth. Nevin and Walsh (1951), showed that physical factors, the degree of separation of the teeth and the shape and width of any contact points or capillarity areas were very important in determining the rate of fall of pH in interproximal spaces. With regard to saliva, both its flow rate and buffering capacity are important in modifying the effects of any cariogenic challenge. Edgar, (1976), found that the neutralising potential of an individual's saliva played a central role in that person's ability to withstand a cariogenic challenge. There are also components of saliva which reportedly affect the protective ability of it. Lassiter et al, (1987) reported that lactoferrin, transferrin, conalbumin and apolactoferrin kill bacteria if not

saturated with iron. Lysozyme and the lactoperoxidase system also exhibit a caries reducing effect. Salivary fibronectin has also been reported to inhibit caries by reducing the adherence of bacteria on the pellicle and therefore reducing the number of bacteria in the mouth (Babu and Dabbous, 1986). The presence of salivary agglutin (Ericson and Rundegren, 1983) has been implicated in increasing the likelihood of caries occurrence, as it favours the attachment of bacteria to the oral epithelium and the tooth pellicle. IgA is the only immunoglobulin to be secreted into the oral cavity in saliva, however, IgG from serum, reaches the mouth via the gingival crevicular fluid (Challacombe et al, 1978), and especially in cases of gingivitis or periodontitis, when the oral tissues will become inflamed, and serum proteins will be exuded into the oral cavity. Aaltonen et al, (1987) reported that there was a link between the amount of serum IgG in an individual's blood and the numbers of Streptococcus mutans in his oral environment. The effects of IgG are to inhibit bacterial adherence, to inhibit bacterial enzymes, bacteriolysis, chemotaxis and opsonization of bacteria. IgA inhibits bacterial adherence to epithelial cells, inhibits bacterial enzymes and has anti-inflammatory activity (Carlsson and Krasse, 1968; Douglas and Russell, 1984; Williams and Gibbons, 1972). There is also a report (Ikeda, et al, 1985), that cites a bacteriocin obtained from Streptococcus mutans which selectively eliminates this bacterium. Some of these factors are available for external modification and others are purely hereditary.

1.5.2. Microflora.

'Plaque is a mat of bacteria which covers human teeth and which is not removed by rinsing with water' (Dawes, 1968). It has been shown that plaque contains in the order of 1 X 10^{11} colony forming units / mg wet weight, and at least 50 distinct species of bacteria (Bowden et al., 1979). There have been many different studies carried out to determine the different bacterial species associated with dental caries. In each case however, different factors concerning each bacterial species need to be considered. For example, the acid production rate of the bacterium, the aciduric capabilities, the acid end-products, the site on the dentition where aggregation is favoured by the bacterium, its extra- and intra- cellular polysaccharide production and whether or not it produces alkaline end-products or is capable of acid utilisation. All these factors come into play when assessing the cariogenic potential of a specific bacterium. The first animal experiments which indicated the necessity of oral bacteria in the aetiology of caries were carried out by Orland, et al in 1954. They found that rats which had no oral bacteria (germ free animals), even when fed a 'highly cariogenic diet', did not develop caries. When these same animals were then infected with bacteria whilst maintaining the same diet, caries resulted. This type of study has indicated that streptococci, eg. S.faecalis, S.mutans, S.salivarius, S.sanguis, S.mitior, and Peptostreptococcus intermedius, A.viscosus, A.naeslundii, A.israelii, L.casei and L.acidophilus are all capable of inducing experimental

caries in gnotobiotic animals. These experiments also indicated that not all acid producing strains of bacteria were capable of inducing caries even in the presence of a fermentable carbohydrate diet. These studies also indicated that there was site to site variation in the ability of microorganisms to produce caries. For example, although the bacteria listed produced caries in tooth fissures, they could not all produce caries on the smooth surfaces or the root surfaces. One problem with these studies is that in a normal situation there may be bacteria present which would modify the ability of the cariogenic bacteria to act, by either preventing adherence to the tooth surfaces or utilising the acids produced by the acidogenic bacteria, eg. Veillonella utilises the lactic acid produced and metabolises it to acetic and propionic acids which are weaker and perhaps less damaging to the enamel (Mikx, et al, 1972; Mikx and van der Hoeven, 1975), as does Neisseria, (Hoshino, et al, 1976). These and other studies have all implicated the efficacy of all strains of S.mutans to produce dental caries but have shown variability in the abilities of the other stains to do the same. Also worthy of consideration in the case of S.mutans, is its ability to produce extracellular polysaccharides when exposed to sucrose. The extracellular polysaccharides greatly increase the volume of plaque present on the teeth and therefore allow greater colonisation of the tooth surfaces by bacterial species. More recently, another, perhaps more important role of the extracellular polysaccharides has been elucidated (Rölla, 1989). It has

been suggested that the extracellular matrix enhances the acid concentration at the plaque / enamel interface by influencing the acid diffusion pattern. Lipoteichoic acid (a bacterial anionic polymer which has a high ionic exchange and buffering capacity) is thought to have a role in this process by influencing the adhesiveness, degree of hydration and the cohesion of plaque. Many different species of bacteria are capable of producing intracellular polysaccharides which they can then metabolise after the cessation of fermentable carbohydrate availabilty, this does not however increase the bulk of the plaque. The acid production rate of some species is also greater than that of others, eg. streptococci tend to have a faster rate of acid production than actinomycetes, and S.mutans has a greater rate of acid production than S.mitis. S.mutans is also able to endure lower pH values than other streptococci as are lactobacilli. Thus, where other bacterial species would die from the effects of the acid environment, these bacteria can continue metabolising the carbohydrate source and continue to produce harmful acid end products.

1.5.3. Diet.

There are various factors which affect the cariogenic potential of a food. These include the pattern and frequency of eating, the food's pH and buffering capacity, its fermentable carbohydrate content as well as its concentration and types of lipids and proteins, its capacity for causing salivary flow and any contents which may affect either the enamel solubilty or the oral

bacteria. Various studies have shown that a food's cariogenic potential is related to the frequency with which it is consumed, the greater the frequency of consumption, the more likely the food is to be cariogenic. A recent study of the between meal intake of food and drinks in 4 year olds (Holt, 1991), showed that there was a significant increase in mean dmft with increasing number of intakes of between meal snacks. Foods which are retained in the mouth for a long period of time are also more likely to have a higher cariogenic potential than those which are quickly swallowed, the food's retentive qualities, stickiness etc., play a role here (Lundqvist, 1952; Gustafsson, et al., 1954). There have been some more recent studies, however, which have indicated that the frequency of eating is less important in developed countries (Rugg-Gunn, et al, 1984; Burt and Ismail, 1986 and Burt, et al, 1988). These workers have suggested that the total sugar consumption as well as the between meal eating pattern is of more importance, although the frequency of eating becomes more important when the overall sugar consumption is low. The sequence of eating is also important, as workers have shown that eating some foods, such as cheese, can modify the acid challenge to the teeth and minimise any harmful effects (Edgar, et al, 1982; Rugg-Gunn, et al, 1975). If the food has an intrinsically high pH or buffering capacity it is also less likely to be highly cariogenic than a food which does not. The fermentable carbohydrate content is of obvious importance, as, if a food does not contain any fermentable carbohydrate there will be nothing on which the

oral bacteria can act to produce acids. There are also modifying factors in foods (which will be discussed in later chapters), which can protect the enamel from demineralisation, eg. fluoride in tea and raisins. Fluoride, itself may be ingested, either in the form of supplements to the diet (drops or tablets) or as a constituent of the water supply. Some foods produce greater amounts of saliva than others (generally related to taste and the amount of chewing required, as the physical act of mastication causes saliva release), and the greater the amount of saliva released the quicker the intraoral pH will return to normal.(Dawes, 1970)

There are various reports citing vitamins and minerals as having a contributory effect on the possibility of caries development. Sortino, et al, (1984) cite zinc as having a cariostatic effect, through the effect of zinc dependent enzymes being involved in the caries process. They also postulate that Vitamin B_6 limits this zinc dependent effect through means as yet undecided, and that Vitamin E has a cariostatic role in the aetiology of caries. Casein reduced caries formation, as did whey protein (Reynolds and del Rio, 1984). Many other foods have been tested and will be discussed more fully in later chapters. Arginine has also been found to reduce pH drop(Duguid, 1987) by decreasing acid production and glucose uptake by perhaps altering the rate of glucose transport or catabolic processes in Streptococcus mutans 10449. Glycerol monolaurin has been found to reduce caries (Lynch, et al, 1983). Sodium trimetaphosphate

has been widely tested in animal studies (Navia, et al, 1968; Mühlemann and König, 1964) and was found to produce reductions in caries of up to 70%. Finn, et al, (1978) tested this substance as an additive to chewing gum, and found that there was a significant reduction in caries increment over the three year study. This finding that sodium TMP and other phosphates could lead to a reduction in the caries increment found in trials led to the addition of phosphates into ready-to-eat breakfast cereals in the USA. These cereals were further tested (Bibby and Weiss, 1970; Wilson, 1979) and it was found that calcium was more important in reducing enamel dissolution than phosphates.

1.5.4. Other Factors.

There has been much discussion concerning the prospect of an anti-caries vaccine. As other bacterial infections can be vaccinated against and the disease reduced or eliminated, why should individuals not be vaccinated against the bacteria which cause caries? The problem lies with the very complex nature of the caries process. Firstly, caries is not produced by one microorganism on its own. There is a whole range of different microorganisms which are capable of metabolising fermentable carbohydrates in the mouth and producing acids which would demineralise the teeth. The different caries resistance of the individuals would also need to be taken into consideration, as different individuals have caries resistance properties unrelated to their ability to resist bacterial infection alone (eg. fluorapatite content of the enamel). The benefit of a caries vaccine also needs to be considered.

Would the oral environment be recolonised by more pathogenic bacteria if the microorganisms currently found in the mouth were eradicated? Caries although it is an unpleasant disease is very rarely life threatening, and whether the development of a vaccination programme would be cost effective is debatable. Side-effects would also have to be non-existant as the disease itself has no inherent risk (Krasse et al., 1987). Another possible route would be to substitute less cariogenic bacteria into the oral cavity in the hope that they would recolonise the mouth and prevent the re-implantation of the more cariogenic strains.

Recent studies, (Higham and Edgar, 1990), have shown the effect on fissure caries in rats, of addition of lactate dehydrogenase (LDH) to their drinking water. LDH is widely distributed in the body tissues, and is responsible for catalysing the conversion of pyruvate to lactate. This study showed that the initiation of fissure caries in rats was delayed by the addition of LDH to their drinking water, and that this enzyme merited further investigation as a possible cariostatic agent.

1.6. Methods Of Assessing Cariogenic Potential.

1.6.1. Human in vivo.

Human clinical trials have been used as a means of determining the cariogenic potential of a substance. In these tests, volunteers are examined prior to the experiment and their tooth surfaces rated as to their previous caries experience. The volunteers are then asked

to eat a test substance over a period of time, either with or without instructions as to their oral hygiene procedures during the experiment. These oral hygiene procedures as well as their background diet are recorded. At the end of the test period, the tooth surfaces are again examined and any differences between the first and second examinations noted. The subjects are then normally given a very thorough prophylaxis and topical fluoride applications to heal any early carious lesions produced by the test food. There are various attendant disadvantages with this technique, although it does provide an essentially in vivo test system. There may be problems with variations in examination criteria, although these can be minimised by using the same examiner throughout (this may be difficult in a large clinical trial however), It may be difficult to mask the effects of the background diet of the individuals which may be exerting a more powerful effect than the food to be tested, Very large numbers of subjects need to be used due to the large subject to subject variation and not least there are ethical considerations in subjecting volunteers to potentially damaging substances. (von der Fehr, et al, 1970; Scheinin, et al, 1975; Gustafsson, et al., 1954).

Workers have used an appliance worn intraorally by human volunteers into which enamel slabs are inserted (Koulourides, et al, 1976;). The subject can then use oral hygiene procedures for his own dentition, but allows the test enamel to be subjected to a cariogenic challenge with the mitigating factors of the

oral environment present. This model overcomes the ethical problems presented by asking volunteers to subject their own teeth to a cariogenic challenge. It is however a time consuming test, and requires that the volunteers be highly motivated and dedicated to the experiment. The subject availability is , therefore limited as is the number of enamel slabs which can be placed in the mouth throughout any one experiment. The conclusions which can be drawn from these experiments also need to be considered in relation to the background diet of the subject. This system is highly appropriate to the techniques of microradiography and microdensitometry, which allow the enamel slabs to be radiographed before, during and after the experimental procedures and is very well controlled as exactly the same area can be analysed throughout the course of the experiment.

Following contact with fermentable carbohydrates, plaque undergoes a well characterised pattern of pH change called the 'Stephan Curve', after R.M.Stephan, who first described it in 1940 (Fig.1.2.). There is a rapid initial pH drop to a minimum which occurs 5 - 10 minutes after the challenge, then a gradual increase over the next 20 - 30 minutes until the pH returns to its resting level. The 'Stephan Curve' produced by each individual is different, and is altered by such factors as salivary buffering capacity and composition, salivary flow rate and viscosity, plaque composition and the actual mechanics of eating, e.g. portion size and mastication time.

There are three major methods of monitoring the pH of

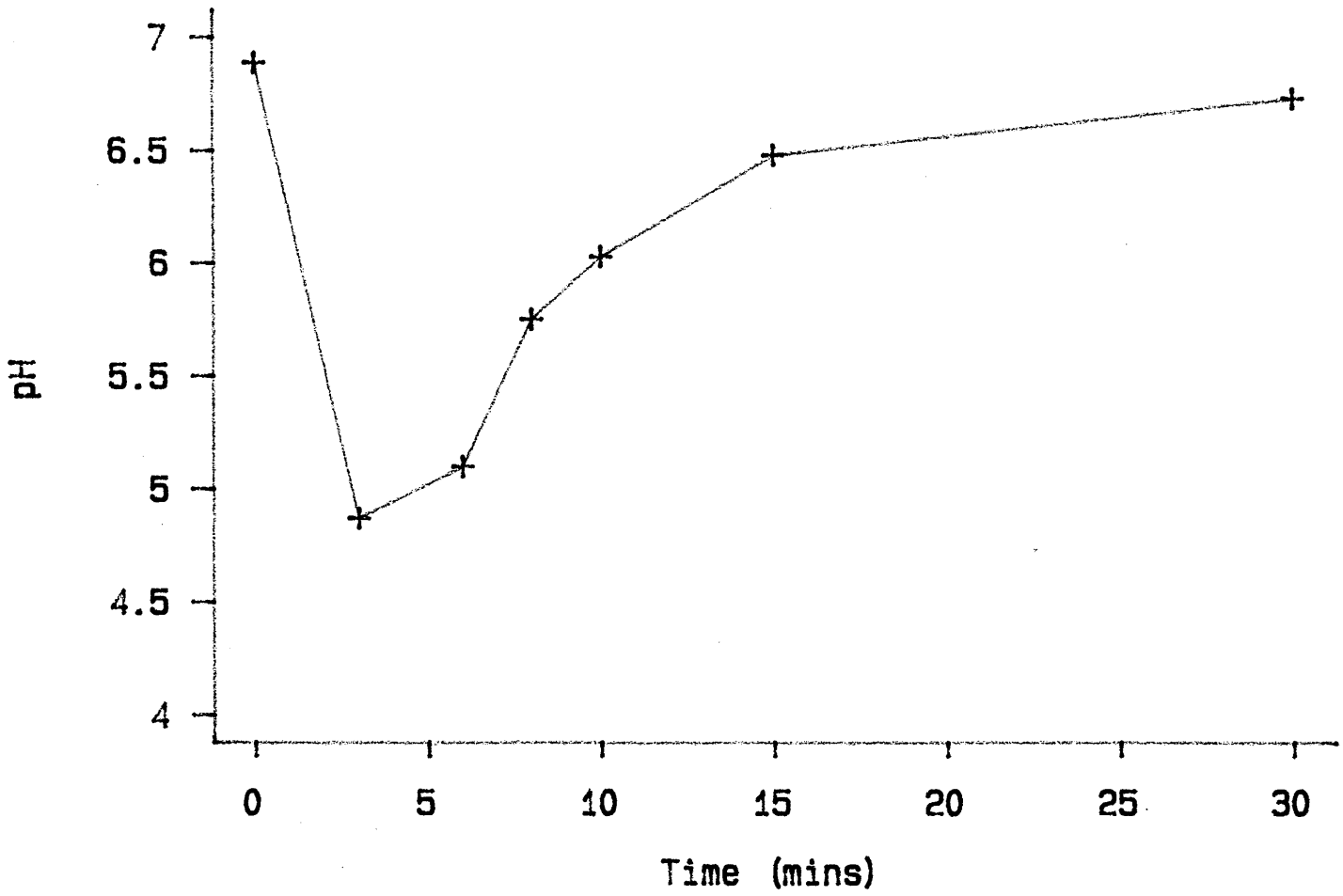

Figure 1.2. Typical 'Stephan Curve'.

a subject's plaque pH, each with attendant advantages and disadvantages: i) The sampling method,

ii) The probing method, and

iii) The indwelling method (both wire and radio telemetry).

i) The sampling method involves taking small samples of plaque from the different sites in the mouth, pooling them, and then measuring the pH with a small cup electrode. The advantages of this method are that it is simple to use and does not require much subject participation. However, it does not give any information on specific site pH change, the plaque quantity is limited (therefore limiting the length of the experiment), and the plaque is not in situ at the time of pH measurement. (Edgar, et al, 1975; Englander, et al, 1956; Geddes, et al, 1977; Rugg-Gunn, et al, 1975 and Rugg-Gunn, et al, 1978).

ii) The probing method uses a pH electrode with a very small tip, which is probed into in situ plaque before and after the test substance is applied. This allows the pH change at individual sites to be monitored and is relatively simple to use. It also demands little subject participation and does not alter the quantity of plaque present, therefore allowing both standardisation and repetition of the pH measurements from the same sites. Again however, the pH measurements cannot be obtained from the most 'at risk' sites, e.g. interproximal surfaces. (Kleinberg, et al, 1981 and Harper, et al, 1985).

iii) The indwelling method involves fitting the subject with a pH electrode built into a hollowed out tooth which

is mounted on a denture assembly. The pH electrode can be either connected to a meter by a wire leading out of the mouth (wire telemetry) or by a tiny radio transmitter (radio telemetry). The plaque is allowed to form over the appliance and the pH is monitored from the internal surface of the plaque. This method allows continuous monitoring of the pH during an experimental procedure and also allows the more caries prone sites to be assessed. It does however, demand a greater degree of subject compliance and requires that the subject has a missing molar tooth suitable for the denture assembly. There are also difficulties with calibration of the electrode due to the need for a plaque layer over the electrode before it can be used. (Graf, 1983; Graf and MÜhlemann, 1966 and Imfeld et al., 1978).

1.6.2. Animal Models.

To overcome some of the ethical problems posed by using human volunteers, various animals have been employed to give an in vivo system for cariogenicity testing. Such animal models have included monkeys (Beighton, et al, 1983, 1984 and 1985), hamsters (Krasse, 1965) and rats (Keyes, 1958; Stephan, 1966). The animal models have various advantages over human models. The systems can be modified in such ways as having gnotobiotic animals and infecting them with specific bacterial strains to control the types of bacteria involved in the experiment and giving their normal diet by gastric tube and only the test substance orally and thus seeing the direct effects of the test food (Bowen, et al, 1983). However, there are

problems in extrapolation of results, especially in the cases of hamsters and rats as the teeth are different from human dentition in their morphology and eruption pattern and the composition of their saliva is different. These experiments are very expensive in terms of animal costs, feeding and specialised care and are very time-consuming. There are increasingly problems with the animal rights lobby as well which have to be considered. The conditions can be very well controlled however, and sufficient numbers of animals can be used to make valid statistical analysis possible. (Bowen, et al, 1980; Orland, et al, 1954). The animal models have proved particularly effective in determining the cariogenic potential of the oral microorganisms, (especially in relation to frequency of consumption) and more recently, gnotobiotic animals have been used to determine the relative cariogenic potential of different foods and the effects on the cariogenic potential of extracellular polysaccharides.

1.6.3. in vitro Fermentation / Artificial Mouths.

These models aim to mimic, as far as possible, most/all of the oral conditions in an in vitro system. Some models exclude saliva to produce a system skewed in favour of demineralisation (Bibby and Mundorff, 1975; Geddes, et al, 1984) while others include it (Bibby, et al, 1951; Curzon et al., 1985). These models incubate enamel with oral bacteria (normally S. mutans), the test substance and, if appropriate saliva (either human or artificial), and monitor various parameters throughout the course of the experiment - such as pH, acid anion production and calcium release. After the experiment the tooth enamel can be

examined and any demineralisation noted. Fluoride or any other factor the workers wish to be considered can also be added to the system, and its effects noted. Of all the experimental methods this is the least related to the in vivo situation, however, the various factors can be extremely well controlled and statistical analysis easily and reliably performed. There are no ethical problems, these methods are relatively cheap and the results can be obtained quickly. The results obtained from these methods do need to be carefully analysed and no exorbitant claims made from them, but they are a very versatile tool in the hands of the worker in the field of cariogenicity testing.

1.7. San Antonio Recommendations.

In San Antonio, Texas, in November 1985, a group of workers concerned with the assessment of the cariogenic potential of food, met to discuss the state of the art concerning the various methods currently employed, to discuss the standardisation of these techniques, and to produce a rational approach as to the way forward. The workers discussed the methods under the headings: Human Plaque Acidity, Animal Caries and De-/Remineralization Models. There was also a group to discuss the integration of these various techniques. They published the results from their discussions, and the recommendations made by the group are meant to allow standardisation of all the various methodologies currently employed in the field, and thus allow different workers to assess more readily, and use the results found by others.

1.7.1. General Recommendations.

The group studying the integration of methods started by giving a clear definition of cariogenic potential: 'The true cariogenic potential of a food can only be established by experimentally determining in humans the extent of tooth decay associated with a given food. Since such experiments are not ethically feasible, scientists must rely on carefully standardized indirect methods for assessing a food's propensity to foster human caries. These indirect methods, therefore, limit themselves to establishing the cariogenic potential of foods without regard to actual food usage or consumption patterns. Accordingly, by cariogenic potential is meant a food's ability to foster caries in humans under conditions conducive to caries formation. To varying degrees, cariogenic potential may be assessed by measuring acid production in dental plaque, by enamel demineralization, and by animal caries formation.'

The group concluded that plaque pH and animal caries models were valid for assessing the cariogenic potential of foods, and that greater reliability and reproducibility could be gained by using these techniques complementarily. They felt that the de-/re-mineralisation models had not been sufficiently tested, but that as this approach was highly desirable as the ethical concerns of animal work increase. These methods would be most promising if further work was carried out. They suggested that a preliminary screening model should be used to obtain foods which would most usefully be tested in more extensive models and thus

optimise the available resources.

They made the following proposals to allow the integration of the various assessment methods into a testing protocol.

1) The de-/remineralization model could in time emerge as a valid and simple test. The Integration Working Group recommends that specific efforts be initiated to refine the demineralization/remineralization models to the greatest extent possible. This recommendation refers to both the in vivo and the in vitro systems.

2) It is recommended that research be continued on the refinements of plaque pH measurement methods so that they can be more effectively used to categorize foods of low cariogenic potential.

3) It is recommended that better methods be developed to permit the testing of foods presently difficult to assess for cariogenic potential.

4) To assist research and development of non- and low cariogenic foods, greater emphasis should be placed on finding simple yet reliable methods for screening foods with respect to cariogenic characteristics.

5) It is recommended that laboratories evaluating the cariogenic potential of foods make greater efforts to incorporate appropriate standard reference foods into their testing procedure.

6) In order that knowledge on the relation between food factors and cariogenicity be extended, further research on this topic is strongly recommended by the Integration Working Group.

1.7.2. Recommendations For De-/Re-Mineralisation Models.

The working group in the De-/Remineralization group made the following Consensus Statement: 'A demineralization / remineralization model is a valuable part of a cascade of tests to establish the cariogenic potential of a test food. We consider that a food which does not produce a pH fall in an in vivo acidogenicity test must be considered non-cariogenic. A fall of plaque pH in vivo caused by a test food is a necessary but insufficient condition for a food to have cariogenic potential, i.e., the pH drop must be of sufficient magnitude and duration for demineralization to occur.'

They then gave the requirements for a de- / re - mineralisation model as:

a) dental enamel as the test material,

b) bacterial challenge,

c) cycling de-/remineralization,

d) saliva incorporation,

e) test foods and controls,

f) pH monitoring,

g) assessment of enamel change, and

h) standardization of the physical food state.

There were also more detailed recommendations with respect to some of these aspects:

a) they recommended that the tests should be carried out on either bovine or human dental enamel, and that the outer surface of the enamel should be abraded by a depth of about 100μm.

b) The challenge to the enamel should be by an artificial

plaque derived from oral bacteria, the preferable strain being Streptococcus mutans, type c. These bacteria should be cultured to early stationary phase in a complex medium plus sucrose, harvested, washed and then applied to the hard tissue.

e) The group suggested that at least two controls should be used in every experiment, and that one should be of high cariogenicity, such as sucrose and that the other be of low cariogenicity, such as sorbitol.

f) pH should be monitored regularly or continuously at the plaque enamel surface or as close as possible to it.

h) It was considered that solid and liquid foods could be assessed in the system. Liquid foods could be added to the tooth and plaque, and solid foods should be finely chopped in order to resemble the state of the food after being chewed. The group concluded that: ' Each of the in vitro and in vivo models, when finalized and characterized will provide a tool which will separate cariogenic foods from non-cariogenic foods, can be used as a good predictor of cariogenic potential, and to assess the capacity for enhancement of remineralization or inhibition of demineralization.'

1.8. Aim Of The Research For This Thesis.

The aim of this thesis was to develop and test an in vitro fermentation system, following the guidelines of the San Antonio conference, which would allow a food to be screened for its applicability for further testing in other test systems. The importance of knowing the cariogenic

potential of a food is becoming more apparent as media and therefore public awareness becomes focussed on healthy living. As more people become aware of their responsibility to their own bodies they look to the professionals to give them the answers to their questions of what is in fact 'good for them'. Whether or not the professional can give the answers to these questions depends on the techniques and resources available. The technique being developed here does not seek to give a definitive answer to whether or not a food is good or bad for teeth, but it does seek to place a food in one of three categories; i) non-cariogenic; ii) intermediate cariogenic potential (less than sucrose but showing positive demineralization); and iii) high cariogenic potential (greater than or equal to sucrose). Only where the food gave a ii) response would there be a need for further testing. Thus the application of this model, when developed, would optimise available resources and thus allow most efficient use of the materials and finance available.

CHAPTER 2

INITIAL PLAQUE pH STUDIES

2.1 Introduction.

The recommendations of the Scientific Consensus Conference on Methods for Assessing the Cariogenic Potential of Foods (1986) included *in vitro* tests using a homogeneous slurry of *Streptococcus mutans* as the bacterial component of a cariogenic challenge. To aid development of this monoculture system a series of experiments with human plaque was performed. The aim of these experiments was to i) find the sucrose concentration which would produce the maximum amount of acid; ii) obtain a pH profile for plaque with sucrose and iii) obtain an acid anion profile for plaque with sucrose. As a result of these experiments, a monoculture system could be developed which as closely as possible, mimicked the results obtained from plaque.

Using a modification of the method of Geddes *et al* (1984) which used human plaque, human tooth enamel and sucrose, in an *in vitro*, 5 day recycling microfermentation system, experiments have been carried out using 0, 5, 10 and 20% (w/v) (0. 146 , 292 and 584 mM) sucrose solutions.

2.2 Materials and Methods.

2.2.1 Plaque Collection.

The plaque used for these experiments was collected from one adult, dentate subject whose plaque had been shown to be acidogenic and who had prior experience of dental caries. The subject refrained from all forms of oral hygiene for 24 hours prior to collection. The plaque was collected using a nickel microspatula, the edges of which had been polished and ground smooth. Plaque was collected from all available smooth surfaces avoiding restorations.

The plaque was 'resting' (Geddes, 1974), i.e. was collected at least two hours after consumption of food or drink. Each experiment required at least 40mg wet weight of plaque, and therefore the plaque was collected daily, pooled and stored at -20°C until this had been attained. (This usually took about 4 days.)

2.2.2. Fermentation.

The pooled plaque was divided into 4 portions which were placed in pre-weighed 0.8ml capped polypropylene tubes and then reweighed and the amount of plaque calculated. Solutions of 0, 5, 10 and 20% (w/v) sucrose of analytical grade (BDH Chemicals Ltd., Poole, Dorset, England.) were made up freshly for each experiment, and 20µl/mg wet weight plaque added to each tube (tooth enamel was omitted for these initial experiments). The tubes were then mixed using a vortex mixer and placed in an orbital incubator (Gallenkamp, Crawley, Sussex, England.) at 37°C. The pH

was taken using a Corning 120 pH meter (Corning Ltd., Halstead, Essex, England.) and 15μl aliquots were removed for isotachophoresis at 0, 10, 30 minutes and 1, 5 and 24 hours. The samples for isotachophoresis were stored at -20°C until required. These experiments were repeated three times.

2.2.3. Isotachophoresis.

The samples for isotachophoresis were placed in a MSE High Speed 18 centrifuge (MSE Ltd., Crawley, Sussex, England.) at 4°C and centrifuged at 18 000g for 15 minutes. The supernatant was then removed and analysed by isotachophoresis (Geddes and Weetman, 1981) and the pellet discarded. (For a description of the theory of isotachophoresis see Appendix 1.) The Tachophor used was a LKB 2127 Tachophor (LKB Instruments, Bromma, Sweden.) fitted with conductivity and ultra-violet detection systems maintained at 12°C (Fig.2.1.). The leading electrolyte was 5mM HCl and 6-amino-n-hexanoic acid with hydroxypropylmethylcellulose (0.2% w/v) at pH 4.2. The terminating electrolyte was n-octanoic (caprylic) acid of approximately 4mM buffered with Tris at pH 5.5. The separation column and injection port were cleaned after each run by rinsing with leading and terminating electrolytes (Fig.2.2.). Standard solutions containing formic, pyruvic, phosphoric, lactic, succinic, acetic and propionic acids, at concentrations of the same order as those expected to be found in the samples, were run through the system and calibration curves obtained by which zone lengths could be transformed into nanomolar concentrations.

Fig.2.1. Tachophor Equipment.

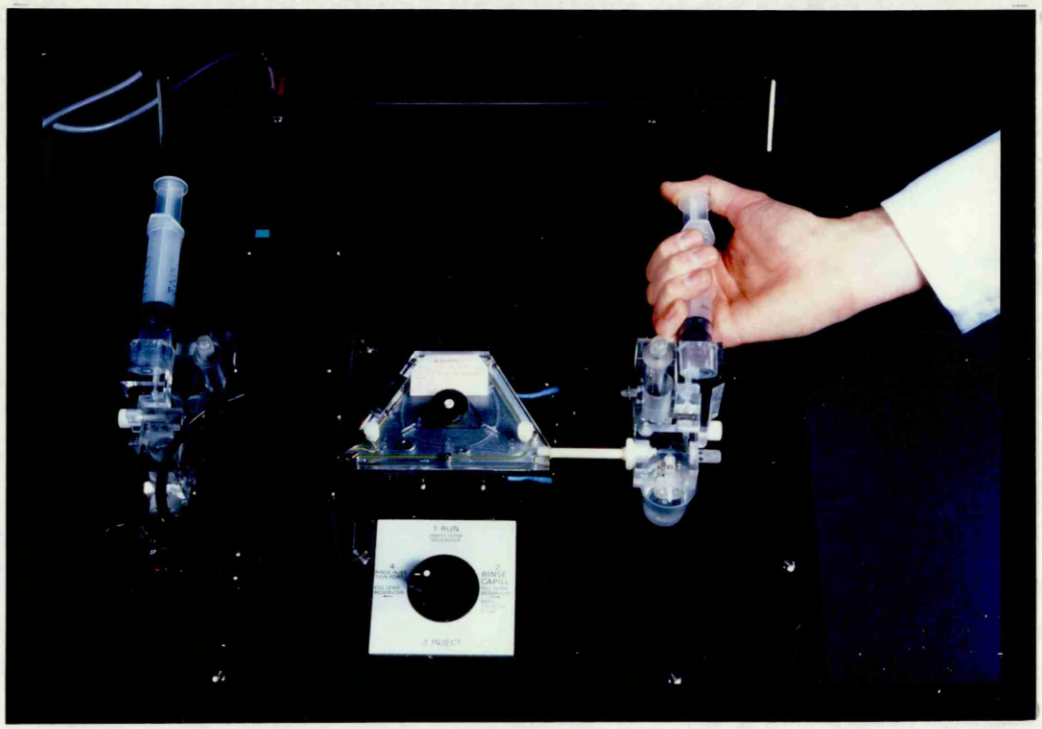

Fig.2.2. Rinse Procedure With Tachophor.

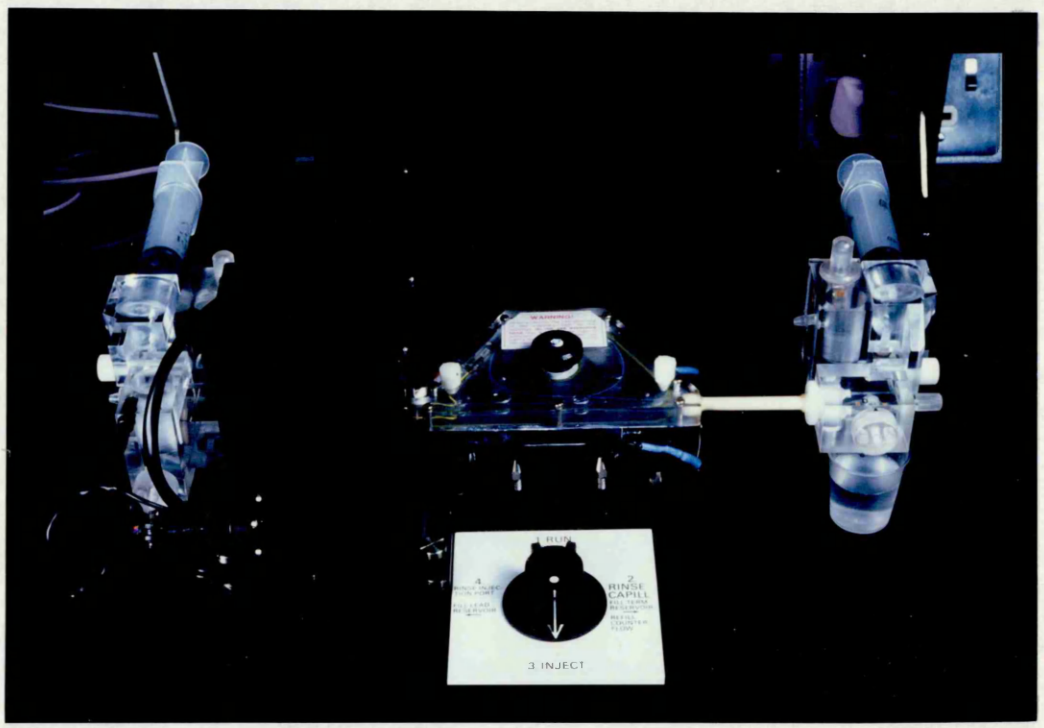

Fig.2.3. Inject Procedure With Tachophor.

(The method for trace analysis can be seen in Appendix 2).

Ten microlitre samples of the plaque/sucrose supernatant were run through the Tachophor at 50μA (Fig.2.3.). The traces obtained were analysed and the amount of each acid calculated in nmol/mg wet weight plaque.

2.2.4. Statistical Analysis.

The results were analysed using a Student's t-test to compare the means of the results from the different concentrations of sucrose solutions, with those of the 0% Sucrose. The results were also analysed using One-way Analysis of Variance (ANOVA), and, where significant, using Scheffé's test for multiple range analysis. The ANOVA and Scheffé's test were carried out using a computer software statistical package. (Statgraphics. ©Statistical Graphics Corporation.)

2.3 Results.

The mean results of the pH profiles obtained in these experiments are shown in Table 2.1. and Fig.2.4.. The mean initial pH of the incubation mixture for all four substrates was 5.7 (S.D.± 0.1.). The 0% (w/v) profile showed no significant change in the pH until between the 5 and 24 hour readings, when the pH increased by 1.2 pH units. In the 5, 10 and 20% (w/v) sucrose solutions there was a rapid initial pH drop, followed by a continuing but less rapid pH decrease. At 24 hours in these cases the mean pH values for each were 4.1, 4.3 and 4.3 respectively. These values were all significantly different ($p<0.001$) from the 0% (w/v) sucrose substrate, using a Student's t-

test and at the 95% Confidence Interval using Scheffé's test. The acid anion profiles are shown in Tables 2.2. and 2.3. and Figs.2.5. and 2.6.. The initial mean acid anion concentration for all four substrates was 111.8 nmol/mg wet weight plaque (S.D.± 26.5). The predominant acid anion was acetate, comprising 56% of the total, with lactate contributing 22% and propionate 12%. The 0% sucrose profile showed a gradual increase in the total acid anions, reaching a concentration of 333.9 nmol/mg wet weight plaque at 24h where the distribution of individual acid anions was acetate ~70%, lactate ~ 3% and propionate ~25%. Following incubation with 5, 10 and 20% sucrose, large increases in the production of acid anions occurred, the 24h mean values being 641.1 (S.D.± 258.2); 656.6 (S.D.± 332.8) and 523.6 (S.D.± 261.9) nmol/mg wet weight plaque respectively. The distribution of acid anions was markedly different from the 0% sucrose, with acetate ~35%, lactate ~60% and propionate ~5%. The values of acid anions were all significantly different ($p<0.001$) using a Student's t-test, from the 0% substrate.

Table 2.1. pH at Various Time Intervals for Each Substrate (mean±SD; n=4).

Time	0%Sucrose	5%Sucrose	10%Sucrose	20%Sucrose
0 min	5.9±0.3	5.8±0.3	5.7±0.4	5.5±0.3
10min	5.8±0.3	5.5±0.1	5.5±0.2	5.2±0.5
30min	5.7±0.2	5.1±0.1*	5.1±0.0*	5.0±0.4*
1 h	5.7±0.2	4.8±0.1*	4.8±0.1*	4.8±0.4*
5 h	5.6±0.2	4.4±0.0*	4.5±0.3*	4.4±0.3*
24h	6.7±0.6	4.1±0.1*	4.3±0.2*	4.1±0.2*

* significantly different from 0% sucrose ($p<0.001$), using a Student's t-test and at the 95% Confidence Interval using Scheffé's test.

Table 2.2 Total Acid Anions (nmol/mg wet weight plaque) Against Time for Each Substrate (mean±SD; n=4).

Time	0%Sucrose	5%Sucrose	10%Sucrose	20%Sucrose
0 min	76.7 ±51.0	114.3 ±93.8	115.0 ±93.8	141.1 ±93.8
10min	114.5 ±38.9	151.0 ±58.7	144.4 ±69.6	119.3 ±43.7
30min	130.4 ±66.4	199.3 ±94.8	202.1±122.0	147.1 ±72.8
1 h	172.5 ±96.5	247.3±110.6	277.3±177.7	178.6 ±54.8
5 h	133.0 ±81.4	288.8±164.5*	405.8±258.4*	284.5 ±97.4*
24h	333.9±184.3	641.1±258.2*	656.6±332.8*	523.6±261.9*

* significantly different from 0% sucrose ($p<0.001$), using a Student's t-test.

Table 2.3. Individual Acid Anions (nmol/mg wet weight plaque) Against Time For Each Substrate (mean±SD; n=4).

i) 0% Sucrose

Time	Lactate	Acetate	Propionate
0 min	18.7±19.7	48.0 ±38.2	12.2 ±4.7
10min	30.9 ±8.7	69.0 ±11.8	12.2 ±2.8
30min	25.2 ±9.4	81.2 ±31.0	14.1 ±4.8
1 h	22.9 ±6.0	120.5 ±36.4	35.2±29.8
5 h	9.7 ±2.6	104.2 ±59.0	18.1±11.1
24h	10.2 ±3.0	238.1±117.1	136.7±82.2

ii) 5% Sucrose

Time	Lactate	Acetate	Propionate
0 min	26.5±13.0	69.9±13.5	13.5 ±5.1
10min	45.5±10.6	80.6±18.9	15.0 ±3.0
30min	83.6±18.9*#	96.7±15.8	18.8±21.7
1 h	120.1±26.8*#	100.1±22.9	18.9 ±6.6
5 h	180.7±81.4*	110.1±43.4	20.6±13.1
24h	418.8±80.6*#	223.4±38.6	37.6±10.8*

* significantly different from 0% sucrose ($p<0.001$), using a Student's t-test.

significantly different from 0% sucrose using ANOVA and thereafter Scheffé's test at the 95% Confidence Interval where appropriate.

Table 2.3. continued.

iii) 10% Sucrose

Time	Lactate	Acetate	Propionate
0 min	23.6 ±9.8	63.5±21.9	11.1 ±5.7
10min	40.2±10.6	74.3±27.7	14.1 ±4.7
30min	74.5±35.6*	89.1±45.2	15.9 ±6.6
1 h	117.8±63.2*#	112.2±58.9	17.7±10.0
5 h	224.8±125.2*#	144.6±62.9	28.3±15.3
24h	400.7±140.7*#	240.8±54.9	44.9±28.2*

iv) 20% Sucrose

Time	Lactate	Acetate	Propionate
0 min	26.5±12.5	66.4±42.8	11.8 ±5.8
10min	37.0 ±3.8	58.7±22.7	12.0 ±2.8
30min	56.8±11.0*	73.0±30.6	11.9 ±1.2
1 h	76.4±14.0*	74.7±21.4	17.9 ±6.5
5 h	141.3±19.1*	117.4±30.9	35.9±29.7
24h	320.2±218.8*	197.5±25.9	43.4±36.8*

* significantly different from 0% sucrose ($p<0.001$), using a Student's t-test.

significantly different from 0% sucrose using ANOVA and thereafter Scheffé's test at the 95% Confidence Interval where appropriate.

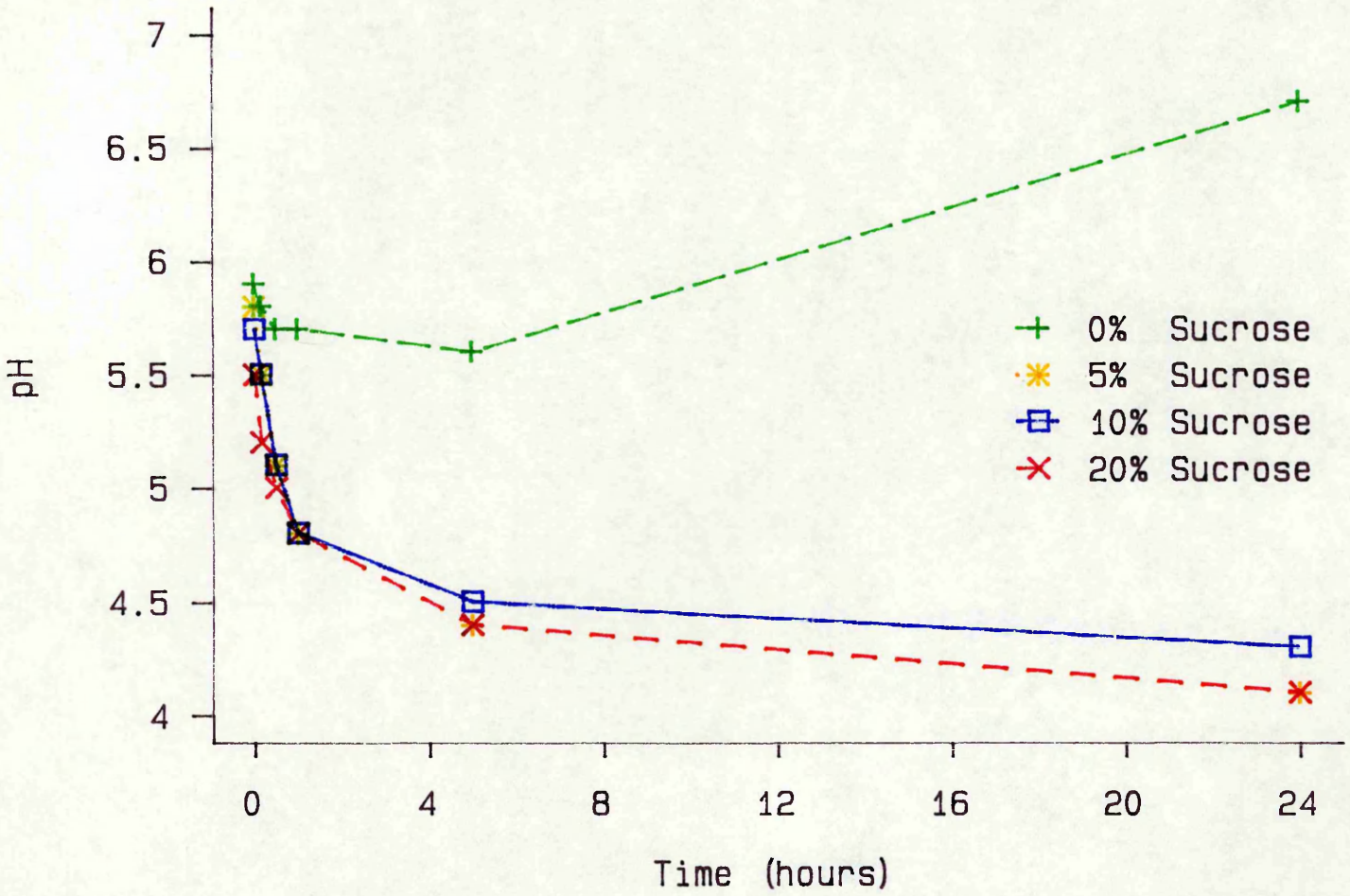

Figure 2.4. Graph of Mean pH Against Time for 0, 5, 10 and 20% (w/v) Sucrose Solutions. (mean; n=4)

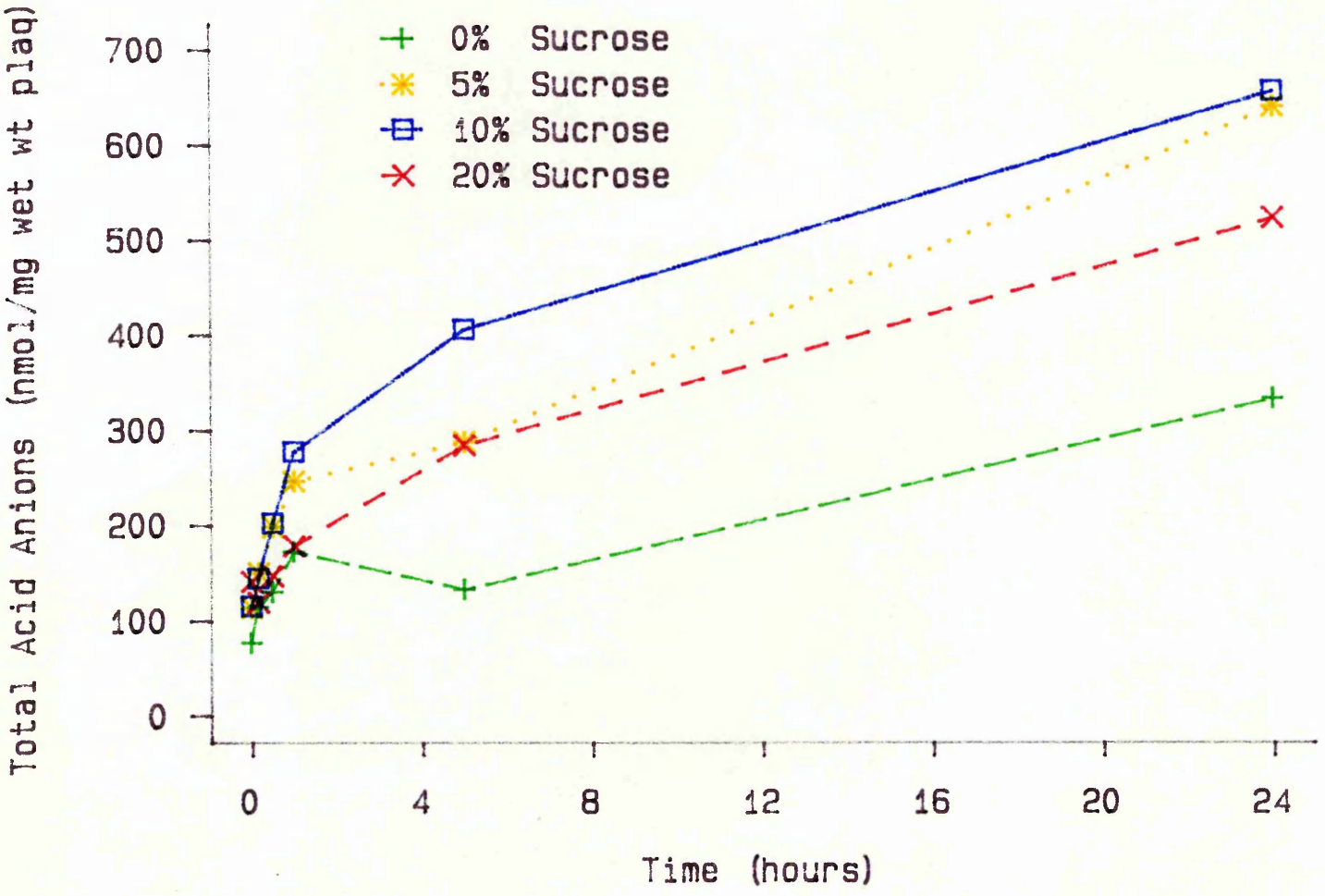

Figure 2.5. Graph of Acid Anions Against Time for 0, 5, 10 and 20% (w/v) Sucrose Solutions. (mean; n=4)

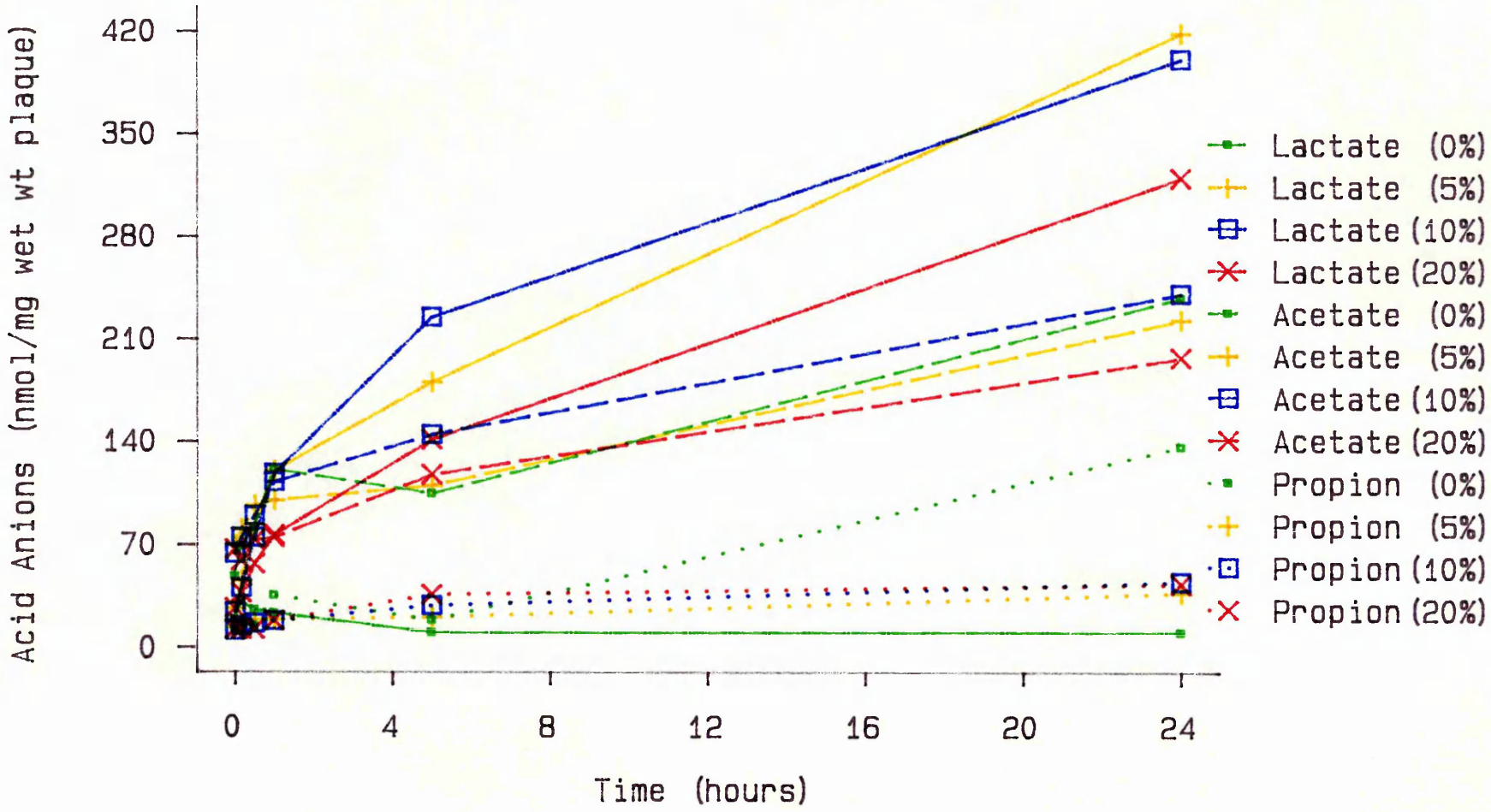

Figure 2.6. Graph of Individual Acid Anions Against Time for 0, 5, 10 and 20% Sucrose Solutions. (mean; n=4)

2.4 Discussion.

The results show that maximal acid production occurred at a sucrose concentration of 5 - 10% (w/v). Many of the components of plaque do not contribute to the acid production, eg. cell debris, food debris and salivary proteins, and some acid production is modified, eg. Veillonella which uses lactate as its energy source (Mikx and van der Hoeven , 1975). Therefore, as the model being developed would be using a single strain of organism and hence without non-acid producing components, it would seem reasonable to use the lower sucrose concentration, ie. 5% (w/v) sucrose.

The pH profiles in Table 2.1. and Fig.2.4.. do not show the classic 'Stephan curve' appearance, as there is no action of saliva nor presence of enamel in this model to clear the sucrose and acid buffer the environment or react with the acid. The profiles do show a rapid pH fall on exposure to sucrose. The 0% sucrose profile also shows an initial drop in pH, although not as pronounced as with the sucrose incubations. It is presumed that this is due to utilisation by the bacteria, of the stored intra-, and extracellular polysaccharides. The rise in pH, towards the end of the experiment, is likely to be due, in part, to bacterial base production and, in part, to cell death and subsequent cell lysis and the release of the alkaline contents. (Yamada and Carlsson, 1973; St.Martin and Wittenberger, 1980; Griffith and Carlsson, 1974.)

Tables 2.2. and 2.3. and Figs.2.5. and 2.6. show the acid anion profiles of the different concentrations of

substrate. In the 0% sucrose case, the main acid produced is acetate, with small amounts of lactate and propionate and very small quantities of formate, pyruvate and succinate. The other profiles show not only much greater total amounts of acid, but also a large shift in the production of lactate. This pattern is consistent with previous studies (Geddes et al, 1984) and similar to that found when incubation occurs in vivo (Geddes, 1975; Distler and Kröncke, 1983; Vrastsanos and Mandel, 1982). This shows the so-called 'Lactate Gate' (Carlsson, 1978).

If microorganisms, adapted to living in a nutrient poor environment are subjected to a rich nutrient supply, they often die. This is caused by an intracellular build-up of glycolytic intermediates - 'sugar-killing'. This situation possibly occurs in the mouth, where the bacteria are subjected to long periods without exogenous substrate, punctuated with short bursts of excess. Therefore, to survive, the bacteria protect themselves in three ways:

i) Regulation of the rate of glycolysis,

ii) Synthesis of intracellular polysaccharides, and

iii) Rapid conversion of pyruvate into lactate.

This third method is the 'Lactate Gate' and allows the rapid formation of large amounts of acid, which in turn leads to loss of tooth mineral and , eventually, caries. Distler et al, (1989), found that members of the S. mutans group were unable to transport lactate into the cell. This suggested an active transport system of lactate and H^+ against a pH and concentration gradient and they postulated that perhaps an increased external lactate concentration

could lead to a regulation of intracellular glycolytic enzymes and therefore a decreased rate of glycolysis. This active transport system has also been reported by Keevil, et al, (1986). Microorganisms which can transport lactate out of the cell even under high external lactate concentrations are at an advantage and therefore this unidirectional transport system of S.mutans would give them an ecological advantage in the oral environment, as described above.

As the concentration of sucrose increases, so does the acid production, until a maximum is reached, which appears from these results to be about the 10% concentration. This result is consistent with that found by other workers (De Boever et al, 1969; Abelson and Pergola, 1984 and Firestone and Navia, 1986a), who found that at concentrations greater than 10% sucrose there appeared to be inhibition of acid production. The concentration and distribution of the various acids examined here, were similar to those seen by other workers (Geddes, et al, 1984.). These workers, using a similar system to the method described here, found acetate concentrations in the range 178-344 nmol/mg wet weight plaque, lactate concentrations in the range 229-562 nmol/mg wet weight plaque and pH in the range 4.4.-4.5 at 24h incubation using a sucrose concentration of 5% (w/v).

This technique provides a basic system which allows the acid production from sucrose to be measured, using the parameters of pH and acid anion production. This system can now be developed to fulfil the requirements of the San Antonio conference by the use of tooth slabs and a homogenous slurry of bacteria.

CHAPTER 3

BACTERIAL PREPARATION

3.1. Introduction.

The 'Scientific Consensus Conference on Methods for the Assessment Of The Cariogenic Potential of Food' (1986), recommended that a homogenous slurry of *Streptococcus mutans*, type c, be used in *in vitro* fermentation systems. They also recommended that they be grown in a chemically defined medium to early stationary phase.

Therefore, it was intended to use a chemically defined medium containing sucrose to cultivate the bacteria, as it was considered that this would more closely mimic the *in vivo* situation (see 3.4. for reasoning), and to use optical density as a measure of cell numbers. Optical density has the attendant disadvantage that it measures not only viable cells but also dead bacteria, extracellular material and cell debris. In order to use the data from this measure, a calibration curve of optical density against viable count is necessary. However, when the bacteria were grown in a medium containing sucrose, the aggregation of the cells was so great that disaggregation of the clumps into single colony forming units could not be obtained without causing cell death, and thus preventing the continuation of the experiment. Therefore, it was decided to grow the bacteria in a glucose containing medium prior to the optical density measurement, and, if necessary transfer the bacteria to a pre-incubation with sucrose before use in the fermentation system .

The aim of these experiments was to i) obtain a

glucose containing, chemically defined medium which was capable of sustaining bacterial growth, ii) obtain growth curves for the bacteria in this medium, and therefore determine the length of incubation required for the bacteria to reach early stationary phase and iii) check to see if the bacteria required a pre-incubation with a sucrose containing medium in order to induce the enzymes necessary for sucrose utilisation.

3.2 Materials and Methods.

3.2.1. Bacteria.

The bacterium chosen for the system was Streptococcus mutans, serotype c, NCTC 10449. This is a bacterium isolated from human caries and was therefore thought to be appropriate for this model. Freeze dried phials of Streptococcus mutans, NCTC 10449, were obtained from the Microbiology Unit of Glasgow Dental Hospital and School. The ampules were opened, reconstituted with 1ml of anaerobic blood broth, and plated out on to Columbia blood agar plates under aseptic conditions, using standard microbiological techniques. The purity of the ampules was then verified using the api 20 Strep diagnostic system (see Appendix 3 for detailed description). Bacteria from a 24h plate culture were removed using a sterile swab, added to 2mls of sterile distilled water and mixed. The first set of wells on the diagnostic strip were then filled with this mixture. The remainder of the mixture was then added to a measured amount of reactive agent provided in the test system and the second set of wells on the strip was filled

with this. This second set of cups, plus the ADH well from the first set, were then covered in mineral oil to exclude air. A plate was taken from the remainder of the mixture to double check the purity of the ampule if required. Both the plate and the diagnostic strip were then incubated in a 5%CO_2/95%N_2 incubator at 37°C for 24h. After 4h the diagnostic strip was removed and the first set of wells examined by i) adding reagents VT_1 and VT_2 to the first well and ii) adding enzymes 1 and 2 to the others. The first set of results could then be read off and the strip returned to the incubator. After 24h, both the plate and the strip were removed, the plate was examined for any evidence of contamination and the second set of results from the wells read. From these results the purity of the bacterial sample was ascertained.

3.2.2. Medium Preparation.

The medium chosen in which to grow the bacteria was that of Terleckyj et al, (1975), as it had already been shown to support the growth of Streptococci. This is a 2% glucose medium the constituents of which are given in Appendix 4. This medium was sterilised using a 45μm filter, poured into sterile 25ml bottles and stored at 4°C until required.

3.2.3. Growth Curve.

In order to determine when the bacteria would be in early stationary phase, a growth curve was constructed. A 24h turbid suspension of bacteria was used as the starter culture and 1ml of this transferred to 25ml of the

chemically defined medium. This was then mixed for 10s on a vortex mixer and incubated at 37°C in an aerobic orbital incubator. Twenty-four, hourly samples of 1ml were aseptically taken and the optical density (E_{540}) obtained using a Pye-Unicam SP8-100 UV/VIS spectrophotometer (Pye-Unicam Ltd., Cambridge, England.) and plotted against time. From this graph the transition from growth to stationary phase could be seen and the optimum incubation time ascertained. This was repeated three times.

3.2.4. Enzyme Induction.

As the bacteria were being standardised in a medium where the carbohydrate was glucose but subsequent experiments were to use sucrose, it was necessary to find the time taken by the bacteria to induce the enzymes necessary for sucrose utilisation.

Bacteria were cultured by transferring 6 blood agar plates of Streptococcus mutans using sterile swabs to 6X25ml bottles of glucose containing medium. These bottles were then incubated in an orbital, aerobic incubator for 18h and centrifuged in a MSE Super Minor centrifuge (MSE Ltd., Crawley, Sussex, England.) for 10min at 700g. The supernatant was then carefully poured off and the pellet washed twice in 135mM KCl (after Marsh, et al., 1982. See 3.4.) before being resuspended in either the chemically defined medium with no carbohydrate or the chemically defined medium with 2% sucrose. The pH was recorded every 30min and a record of pH fall (and therefore carbohydrate utilisation) against time obtained.

3.2.5. Bacterial Preparation.

Bottles of sterile defined medium were brought to 37°C in an aerobic incubator, after which sterile swabs were used to remove all the colonies from two blood agar plates which were then suspended in the medium. The turbid suspensions were then placed in an aerobic orbital incubator for 20h. The bacterial suspension was then centrifuged at 700g for 10min and the supernatant discarded. The bacterial pellet was then washed in 135mM KCl solution and centrifuged at 700g for 10min. The supernatant was again discarded and 500μl of KCl solution added and the mixture vortexed for 10s. The mixture was then transferred to a preweighed 0.8ml polypropylene capped centrifuge tube using a 1ml pipette. The capped tube was then centrifuged at 8000g for 10min and the supernatant discarded. The tube was reweighed and the weight of bacteria calculated. This pellet was used as the 'artificial plaque' for the experiments.

3.2.6. Statistical Analysis.

The statistical analyses performed were the same as those described in 2.2.4.

3.3. Results.

3.3.1. api 20 Strep.

The api 20 Strep system gives the following profile for S.mutans NCTC 10449:

VP	HIP	ESC	PYRA	αGAL	βGUR	βGAL	PAL	LAP	ADH
+	−	+	−	+	−	−	−	+	−

RIB	ARA	MAN	SOR	LAC	TRE	INU	RAF	AMD	GLYG	HAEM
−	−	+	+	+	+	+	+	−	−	−

With this profile being obtained the probability of the bacterium investigated being S.mutans is 99.99%. This profile was always obtained with the bacteria used before using them in the test system.

3.3.2. Growth Curve.

The growth curve shown in Table 3.1. and Fig.3.1. shows the typical phases of a bacterial growth curve (similar growth curves were shown by all four suspensions). In this case, lag phase lasted 13 – 14h. The exponential phase lasted 9h, after which the bacteria went into stationary phase.

3.3.3. Enzyme Induction.

Table 3.2. and Fig.3.2. shows a graph of pH against time for the bacteria which had previously been grown in a glucose containing medium, which were then transferred to either a medium with no carbon source, or one containing 2% sucrose. The pH was monitored every 15min as a measure of the acid production, and therefore utilisation of the sucrose. If the bacteria had to make the necessary enzymes

for sucrose use, we would have expected to see a delay in pH decrease from the sucrose metabolism and the pH of the 0% and the 2% sucrose tests keeping pace until the enzymes were induced, at which point the pH of the 2% sucrose test would be expected to fall much more rapidly. In this case, however, the pH of the 2% case fell much more quickly than that of the 0% sucrose from the outset. The difference between the two conditions was statistically different at 30min ($p<0.01$) using a Student's t-test.

Table 3.1. Optical Density (E520) Against Time For *Streptococcus mutans* Grown in a 2% Glucose Chemically Defined Medium (mean±SD; n=4).

Time	O.D.(E520)
0 h	0.006(0.000)
1 h	0.010(0.002)
2 h	0.008(0.001)
3 h	0.010(0.001)
4 h	0.011(0.001)
5 h	0.014(0.002)
6 h	0.014(0.001)
7 h	0.018(0.004)
8 h	0.021(0.001)
9 h	0.021(0.004)
10h	0.024(0.001)
11h	0.025(0.001)
12h	0.026(0.002)
13h	0.031(0.001)
14h	0.044(0.003)
15h	0.074(0.003)
16h	0.136(0.003)
17h	0.262(0.010)
18h	0.516(0.035)
19h	0.951(0.052)
20h	1.398(0.038)
21h	1.706(0.012)
22h	1.877(0.005)
23h	1.984(0.011)
24h	2.030(0.010)

Table 3.2. pH Against Time For Streptococcus mutans Grown in Glucose Containing Medium, Placed into Either Sucrose Containing or Non-Sucrose Containing Chemically Defined Medium (mean±SD; n=4).

Time	2% Sucrose	0% Sucrose
0 h	6.5(0.1)	6.5(0.0)
0.5h	6.4(0.0)	6.5(0.0)
1 h	6.2(0.1)	6.5(0.0)
1.5h	6.2(0.2)	6.5(0.0)
2 h	6.1(0.2)	6.4(0.0)
2.5h	6.0(0.1)	6.4(0.0)
3 h	5.9(0.2)	6.4(0.0)
3.5h	5.8(0.1)	6.4(0.0)
4 h	5.7(0.1)	6.4(0.0)
4.5h	5.7(0.2)	6.4(0.0)
5 h	5.6(0.1)	6.4(0.0)
24h	5.0(0.3)	6.5(0.0)

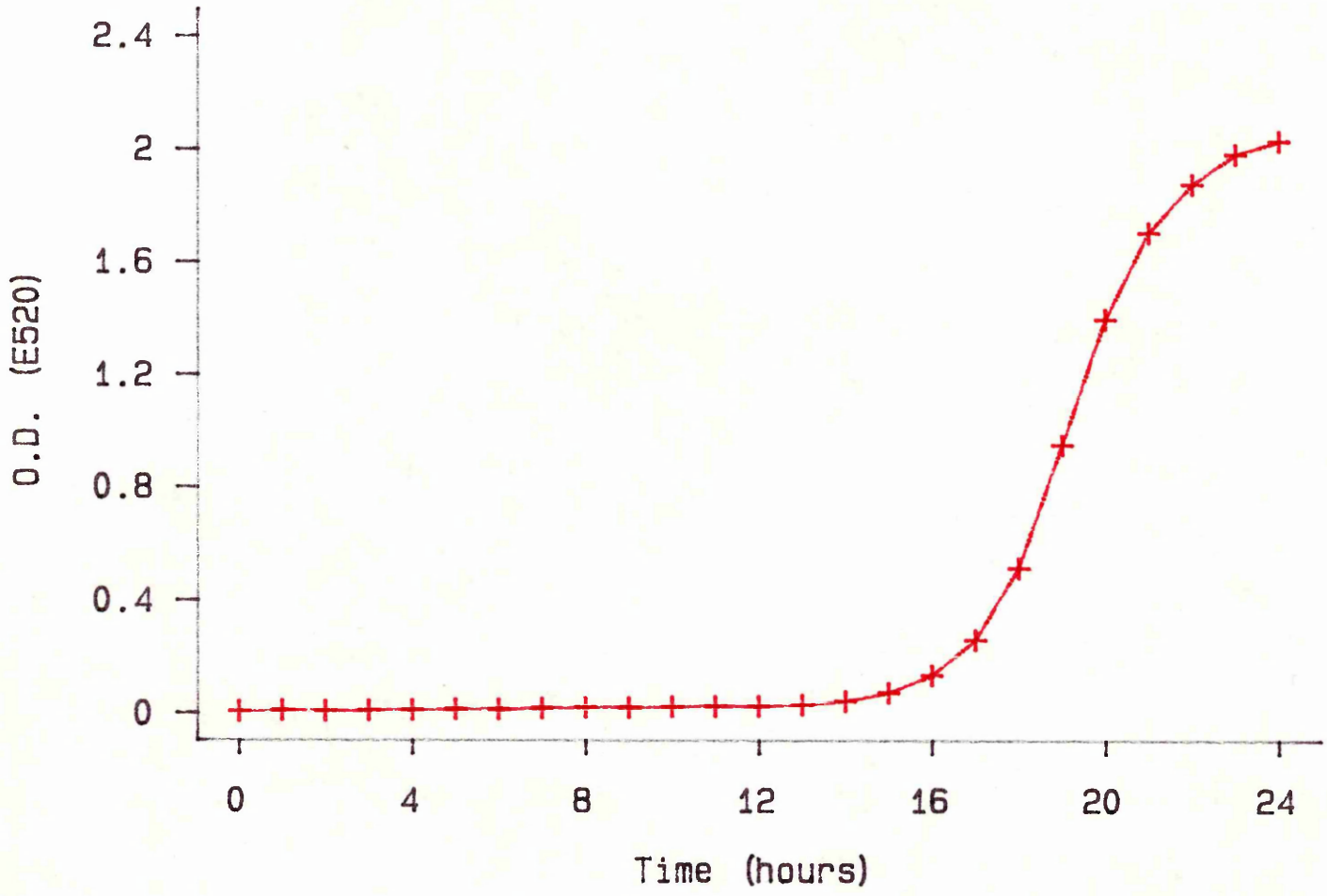

Figure 3.1. Growth Curve for Streptococcus mutans NCTC 10449 Grown in Defined Medium with 2% Glucose. (mean; n=4)

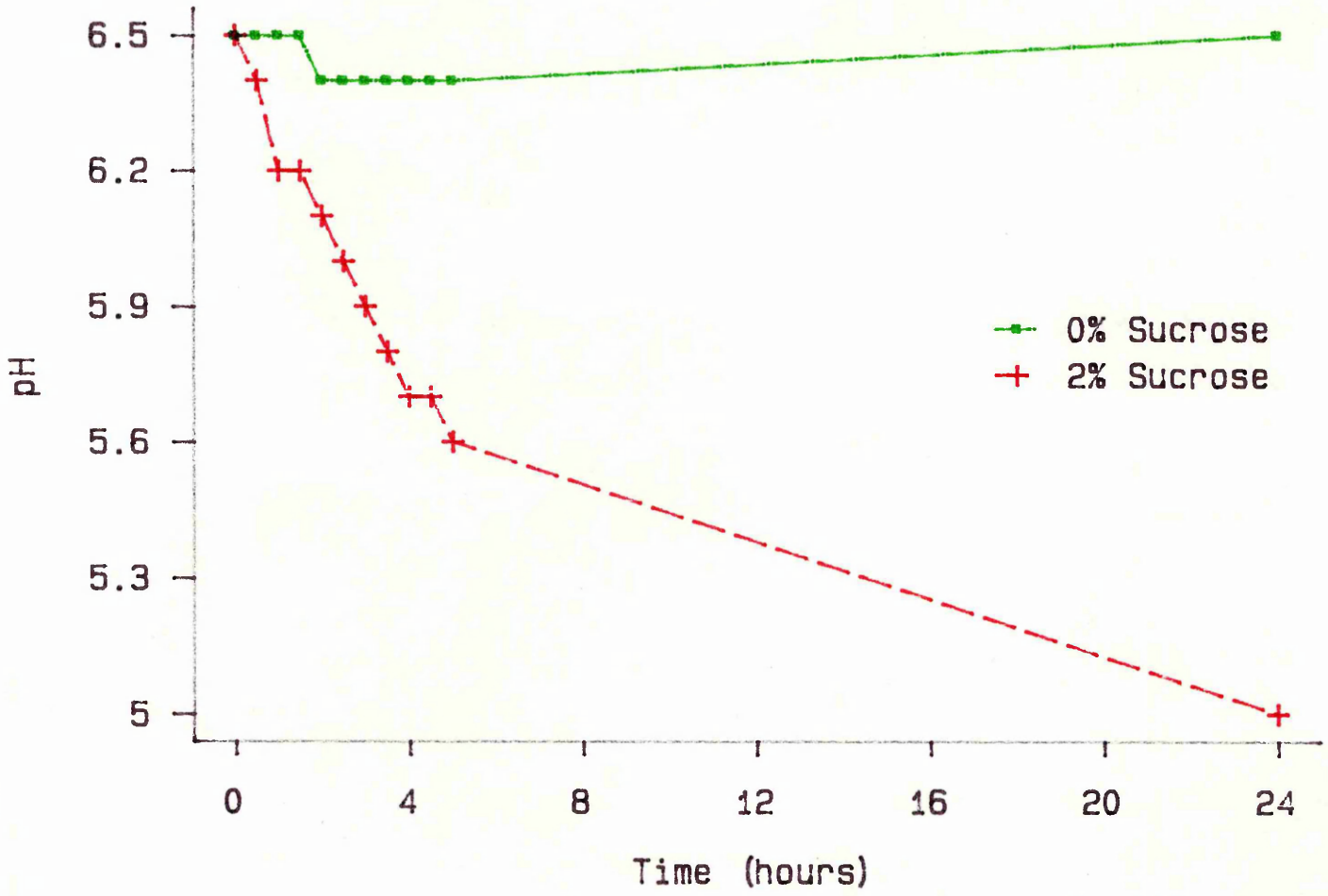

Figure 3.2. Graph of pH Against Time for Streptococcus mutans Grown in 2% Glucose Medium and Then Transferred to Either 2% Sucrose or 0% Sugar Medium. (mean; n=4)

3.4. Discussion

Fig.3.1. shows the growth curve of *S. mutans* in the chemically defined medium. Initially, the bacteria are in LAG PHASE, at which stage they go from being growth limited, by lack of nutrients, and/or build-up of waste products to being capable of replication. At this point the bacteria go into EXPONENTIAL PHASE. During this phase the bacteria replicate very quickly and the growth rate decreases only when some nutrient(s) in the culture medium become(s) depleted and end-products accumulate to a level which causes inhibition. This begins STATIONARY PHASE, during which the cell numbers do not increase and the cells can survive until the nutrient content of the medium becomes too low at which point CELL DEATH occurs. In this case the lag phase is longer than one would normally expect to see. This can perhaps be explained by the chemically defined medium, as although it contained all the necessary nutrients for growth of the bacteria, was not as rich in glucose and other constituents as other nutrient broths in which the bacteria had been cultured. It would perhaps take the bacteria longer than usual therefore to progress to the replication state. From this graph, it would appear that a 20 - 22h culture would therefore be in the area of growth required by the recommendations of the Consensus Conference.

Most individuals eating a 'normal' Western diet could expect that their oral bacteria would have all the necessary enzymes for sucrose utilisation. Sucrose is used very commonly in our refined diets. Even when not included

in obvious forms such as biscuits, chocolate, sweets, sugar in tea etc., the food manufacturing companies regularly add sucrose to such products as tomato soup and baked beans to add taste and texture. Even such unexciting products as 'All-Bran' have added sucrose on their list of ingredients!

Following the work of Marsh et al, (1982), in which they found that the sodium ion inhibited the acid production in S.mutans and S.sanguis, 135mM KCl was used for washing the bacteria. These workers found that the potassium ion stimulated acid production and concluded that the sodium ions are excluded from the bacterial cells at the expense of membrane energy, the protonmotive force, and this energy could otherwise be used to transport sugars across the cell membrane. As a result of this research it was decided to use 135mM KCl as the washing agent in the experiments, and not the usual 135mM NaCl.

3.5. Summary of Experimental Protocol

The protocol for bacterial preparation was thus determined to be the following:

i) Open freeze-dried ampoule of S.mutans, add anaerobic blood broth and plate out (X2) on Columbia blood agar. Incubate in CO_2 incubator for 24h.

ii) Check purity of plates using the api 20 Strep system and plate out required number of plates for experiment on Columbia blood agar. Incubate overnight in CO_2 incubator.

iii) Using sterile swabs, transfer all the colonies from the agar plates to the bottles of 20ml defined medium at 37°C in the ratio 2 plates to 1 bottle. Incubate aerobically at 37°C with shaking, for 20 - 22h.

iv) Centrifuge half the bottles at 700g for 10min. Discard supernatant and resuspend the pellet in the uncentrifuged medium plus bacteria, bottle.

v) Take 4ml of this for optical density readings and centrifuge the remainder at 700g for 10min. Discard supernatant and wash pellet in 135mM KCl before centrifuging as before.

vi) Discard supernatant and wash again as in v). Recentrifuge.

vii) Discard supernatant and add 500μl of 135mM KCl and mix. Transfer mixture to preweighed 0.8ml centrifuge tubes, and centrifuge at 8000g for 10min. Pour off supernatant and weigh pellet plus centrifuge tube.

viii) Add enamel slabs and substrate in the ratio 3μl/mg wet weight bacteria and take pH readings and 30μl samples for isotachophoresis at 0, 10, 30min, 1, 5 and 24h.

CHAPTER 4

ENAMEL PREPARATION

4.1. Introduction.

The delegates at the 'Scientific Consensus Conference on Methods for the Assessment of the Cariogenic Potential of Food' (1986), recommended that any *in vitro* demineralisation models being developed should use either abraded human or bovine enamel. They suggested the use of abraded enamel as *in vitro* experiments have shown that demineralisation is faster in enamel that has been abraded than in untreated enamel (von der Fehr, 1967). This is the result of various factors: lower mineral content, lower concentration of fluorapatite, wider diffusion pathways and different crystal orientation.

Bovine enamel is about three times more susceptible to artificial lesion formation than human enamel (Featherstone and Mellberg, 1981) and also more readily obtainable, for these reasons it was decided to use abraded bovine enamel in the development of this model. In the analysis of demineralisation of the enamel, it was decided to use microradiography and microdensitometry to evaluate if lesions were formed during the course of the experiment and to see at least qualitatively, if not quantitatively, the severity of the lesion formation. A colorimetric calcium assay was also employed to determine the amount of calcium released, and thus the amount of the mineral loss from the enamel could be estimated.

4.2. Materials and Methods.

4.2.1. Bovine Teeth.

Fresh bovine teeth were obtained when required from a local butcher. The central two incisors were removed (also the two lateral inscisors where they were large enough), and the teeth washed well to remove any tissue debris or other extraneous matter. The teeth were then cut, using a dental drill (Millbro, Epsom, England.) fitted with a diamond cutting disc, into eight, nine or ten slabs, depending on the size of the tooth. The enamel face was then abraded, using a slurry of bauxite powder (Raymond A. Lamb, London, England.) and double-distilled, de-ionised water on a ground glass plate (Shandon Southern Products, Runcorn, England.), by 100μm ± 10μm which was measured using a micrometer. The slabs were then examined for any signs of damage, and, if none was present given two coats of an acid resistant varnish (Max Factor Extra Wear, Max Factor, London, England.) leaving an enamel window of ~2mm X 2mm. The window size was measured after the varnish had dried overnight using a X10 magnification eyepiece with integral Vernier scale (E.Leitz (Instruments) Ltd., Luton, England.). These slabs were then used in the experiment. After each daily exposure to the experimental challenge, the slabs were removed from the capped centrifuge tubes, washed using double-distilled, de-ionised water and examined to ensure that none of the nail varnish had flaked off. If this had occurred the varnish was repaired. The slabs were then replaced into tubes containing a fresh

bacteria/substrate mixture.

4.2.2. Calcium Assay.

Using the 30μl aliquots removed at 0 and 24hours for acid anion and calcium analysis, a modification of the Sigma Diagnostic Kit colorimetric assay using o-cresolphthalein complexone was used (Sigma Diagnostics, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178, U.S.A). A calcium assay solution was prepared by mixing equal volumes of the calcium binding reagent (o-cresolphthalein complexone, 0.006%, 8-hydroxyquinoline, dimethyl sulphoxide [DMSO] and surfactant) and calcium buffer reagent (2-amino-2-methyl-1-propanol, 450mmol/l) in a plastic container. This solution was then stored in a refrigerator at 4°C for no longer than 1 week.

One ml of calcium assay solution was added to matched cuvettes labelled TEST and STANDARD and the absorbance (A) against water read at 575nm using a Pye-Unicam SP8-100 UV/VIS spectrophotometer (Pye-Unicam Ltd., Cambridge, England.). Ten microlitres of a calcium standard solution (Calcium 10.0 mg/dl, and Phosphorus 5.0 mg/dl) was added to STANDARD and mixed immediately, and 10μl of sample was added to TEST and mixed immediately. The absorbance of TEST and STANDARD vs water as reference was then read within 10min.

The calculation of the calcium concentration from the colorimetric assay and the subsequent treatment of the results obtained from it was carried out as follows.

Calculation:

$$\text{Calcium(mg/dl)} = \frac{\text{FINAL } A_{TEST} - \text{INITIAL } A_{TEST}}{\text{FINAL } A_{STANDARD} - \text{INITIAL } A_{STANDARD}} \times 10^{*}$$

*Concentration (mg/dl) of calcium standard solution.

As can be seen, the results obtained give the calcium concentration in mg/dl. To obtain the concentration in mmol/l, the result is divided by a conversion factor of 4.008, which is the number of mg per mmol X 10. This result was then divided by the area of the enamel window, to give the calcium concentration in mM/mm^2.

4.2.3. Section Preparation.

At the end of the experiment, the enamel slabs were removed from the bacteria/substrate mixture and washed with double-distilled, de-ionised water. They were then examined for any damage to the varnish which could have left areas of the tooth slab unintentionally exposed. The slabs were then sectioned by mounting them on a specially designed chuck using 'Loc-tite' adhesive (Loc-tite (U.K.) Ltd., Welwyn Garden City, England.), which was then mounted in a saw microtome (E. Leitz (Instruments) Ltd., Luton, England.). Sections were then cut to a thickness of 250μm, perpendicular to the surface, with the blade at the slowest approach speed and with the dentine being cut first to minimise the risk of damage. The sections were then

Fig.4.1. Digital Micrometer.

hand-ground to approximately 100μm with a slurry of bauxite powder and double-distilled, de-ionised water on a ground glass plate (Shandon Southern Products, Runcorn, Cheshire, England.). The exact thickness was measured along the length of the section using a digital micrometer (Mitutoyo, Tokyo, Japan) (Fig.4.1.), and the similarity of the measurements checked to ensure that the sections were planoparallel. The section thickness was required to be 100-120μm to allow adequate grey level ranges for the microradiographic assessment.

4.2.4. Microradiographic Assessment.

The sections were then placed on cling film, next to an aluminium stepwedge, covered with cling film and mounted on Kodak high resolution plates (Type 1A) (Eastman Kodak Company, Rochester, New York, USA.) and microradiographed using Cu Kα Ni filtered radiation, using the parameters 30mA, 20kV, 300mm target focus distance, (using an Enraf Nonius generator) for 20min.

4.2.5. Microdensitometric Assessment.

Microdensitometry was performed using a Leitz/ASBA image analyser to quantify mineral content (Strang et al, 1987) (Fig.4.2.). The radiograph was positioned on the microscope stage and the saturation level of the ASBA determined. The shutter was then positioned to block out all the light entering the lens and the 'black level' obtained. The radiograph was then moved so that areas of 64X64 pixels (192X192μm) were sampled from each of the 8 steps in the aluminium stepwedge, the average grey level

Fig.4.2. Microdensitometry Arrangement.

corresponding to the thickness of the step calculated, and transferred to the BBC-B microcomputer.

The section was then positioned so that the field of interest was in view and horizontal on the ASBA monitor. This image was digitised and transferred to the BBC-B microcomputer. The computer then colour coded the grey levels and displayed a colour image of the enamel on the screen. The area of interest on the enamel surface was located and the computer calculated the average densitometric profile of this area, after which a fourth order polynomial was fitted to the grey levels and the percent volume mineral obtained using the equation derived by Angmar et al, (1963). The profile and any other relevant data were stored on disc for further analysis. The software for both the ASBA and the BBC-B computers was written by Dr.R.Strang and Mr.I.P.A.Macdonald (Glasgow Dental Hospital and School).

4.3. Results.

As this chapter is concerned with the methods used to prepare the enamel slabs and analyse the effect on them, there are no quantitative results.

4.4. Discussion.

The natural lesion is normally triangular when seen in longitudinal section, and consists of 4 distinct areas (Silverstone, 1973) when viewed with polarized light microscopy and microradiography. The surface of the lesion is intact and well mineralised, having a pore volume of <5%. Underneath this there is a demineralised body of the

lesion which contains a banded pattern parallel with the striae of Retzius. Under this are the dark zone and the translucent zone, which are more difficult to observe unless imbibed in a clearing agent. The dark zone has a pore volume of 2 - 4% and is present at the advancing front of the lesion, and it perhaps is a result of de- and re-mineralising processes. The translucent zone, which when observed, occurs at the very front of the advancing lesion, and has a pore volume of slightly more than 1%. These different zones are shown microradiographically as changes in loss of mineral from the enamel.

The use of abraded bovine enamel allows the experiment to proceed much more quickly than it would if intact human enamel were used. Pearce (1983) used different artificial *in vitro* caries systems to simulate incipient caries in abraded bovine enamel, and he concluded that intact human enamel behaved differently from abraded bovine enamel, possibly due to structural differences and the presence of the chemically distinct natural outer layer. As this technique is to be used as a screening test for foodstuffs, in order that the foods of interest may be determined before subjecting them to more time-consuming and more expensive models, faster production of lesions is an advantage, but the differences must be remembered when analysing the results obtained from this method. It must also be borne in mind when interpreting the results that there was no added fluoride to aid remineralisation and no added buffers, such as would be found in saliva. As a result, the enamel, although pH cycled every 24h, was

subjected to a low pH for a longer continuous time period than would normally occur *in vivo*, except perhaps for fissures and unrestored cavities.

It has been suggested that this method does not produce lesions consistent with the pattern normally seen when human teeth are subjected to *in vitro* acid gel treatment to produce 'artificial' lesions, and that the demineralisation pattern seen is a result of the extreme nature of the acid attack produced by the method (ten Cate and Duijsters, 1981). Certainly, acid etching does occur, but the microradiographs do show the classic pattern, (see Fig.5.5.) (as described earlier), of demineralisation in the lesions formed by this method. The surface zone is perhaps not as marked as in other methodologies, but this could be due to the deliberate mechanical removal of the surface layer or the lack of remineralisation phases in this treatment. In the initial stages of development, there were problems in determining how many daily cycles the enamel slabs should be put through. The number of cycles had to be large enough to allow the formation of lesions but not so large that tissue damage was too great to allow the sectioning procedures. Five daily cycles was tried initially, as this was the number of cycles used by Geddes *et al* (1984) using human enamel. However, abraded bovine enamel is more susceptible to acidogenic attack, and, after 5 days the outer layers of the exposed enamel surface were flaking off. Two days was found to be a more suitable time scale which better mimicked natural lesion formation.

Due to the nature of the experimental set-up, the

microradiography and microdensitometry could not be used quantitatively.

This was due to two main reasons: i) The basis of the technique of microradiography is the Angmar Equation (Angmar et al, 1963.):

$$\% \text{ Volume Mineral} = \frac{52.77 t_a}{t_s} - 4.54,$$

t_a = thickness of aluminium stepwedge, and

t_s = thickness of section.

This equation is based on calculations for human enamel, and may be therefore only an approximation for bovine enamel, which has a greater organic component than human enamel (Spitzer and ten Bosch, 1975).

ii) In order to obtain a correct result for the mineral loss from the enamel slabs a control is needed. This technique is so precise that the preferred control is to use exactly the same area before and after the experimental procedure. As this method uses tooth slabs, which are not sectioned until the end of the experiment, this obviously is not possible. The nearest approximation for a control was therefore to use an area of enamel adjoining the experimental window but protected by the nail varnish.

This technique was very good qualitatively, as it provided an excellent visual representation of the enamel surface and gave a numerical feel to the demineralisation taking place. Qualitatively the results obtained using the microradiography and microdensitometry were in good agreement with the quantitative data obtained for calcium

release using the colorimetric assay.

CHAPTER 5

CARIOGENIC POTENTIAL OF DIFFERENT SUGAR SOLUTIONS

5.1. Introduction.

The 'Scientific Consensus Conference on Methods for Assessing the Cariogenic Potential of Foods' (1986), recommended that solutions of sucrose and sorbitol be used as positive and negative controls respectively in any *in vitro* demineralisation model. The aim of the first set of experiments was to evaluate the performance of sucrose and sorbitol as positive and negative controls respectively in this *in vitro* test system. Secondly, a further set of sugar solutions, found to have differing cariogenic potentials in other test systems was tested to ensure that this test system adequately differentiated low, medium and high acidogenic challenges.

5.2. Materials and Methods.

5.2.1. Sucrose, Sorbitol and Water Incubations.

Three tooth slabs were prepared as described in 4.2.1.. Twelve Columbia blood agar plates were plated out with *Streptococcus mutans*, NCTC 10449 under aseptic conditions and incubated overnight in a 95% N_2/5% CO_2 incubator at 37°C. After the overnight incubation the plates were visually checked for any signs of contamination and, if none were found the colonies were removed using a sterile swab and suspended in pre-warmed (to 37°C) bottles of chemically defined medium, two plates to each bottle of medium. The bottles were then placed in an aerobic orbital

incubator at 37°C for 20h. Following the incubation, the bottles were removed from the incubator and centrifuged in a MSE Super Minor centrifuge (MSE Ltd., Crawley, Sussex, England.) for 10min at 700g. The supernatant was then carefully poured off and the pellet washed twice (centrifuging between) with 135mM KCl, before being resuspended in 0.8ml KCl solution, placed in 0.8ml capped, polypropylene tubes and centrifuged at 18 000g and 4°C for 15min. The supernatant was then decanted and the pellet retained for subsequent use. This pellet of Streptococcus mutans was incubated with an enamel slab and either 5% (w/v) sucrose (146 mM), 5% (w/v) sorbitol (274 mM) solutions or double-distilled, de-ionised water as a control, in the ratio 3µl substrate/mg wet weight bacteria, (typically bacteria - substrate volume is 0.5 - 0.6ml) at 37°C for 24h with continuous shaking. The enamel slabs were placed in fresh bacteria : substrate incubation mixture each 24h for 2 days. The pH was recorded and 30µl aliquots taken for acid anion and calcium analyses at 0, 10min, 30min, 1h, 5h and 24h. These aliquots were centrifuged at 18 000g and 4°C for 15min and the supernatant used for the analyses. This was stored at -20°C until required. The analyses of the acid anions was carried out as described in Chapter 2.2.3., the total calcium as described in Chapter 4.2.2. and the enamel analysis as described in Chapter 4.2.3., 4.2.4. and 4.2.5..

The 2-day tests were each run 4 times, giving 8, 24h sets of pH, acid anion and calcium results and 4 sets of tooth slabs for microdensitometric analysis as this measurement could only be carried out at the end of each

test.

5.2.2. Lactose, Galactose and Xylitol Incubations.

The common dietary sugars lactose and galactose and the sugar alcohol xylitol were tested. The incubations and analyses were carried out as described in Chapter 5.2.1., and included 5% (w/v) sucrose and 5% (w/v) sorbitol as positive and negative controls respectively. The test solutions were 5% (w/v) lactose (146 mM), 5% (w/v) galactose (277 mM) and 5% (w/v) xylitol (328 mM). Five tooth slabs were prepared and twenty Columbia blood agar plates cultured.

5.2.3. Statistical Analysis.

The statistical analyses of the results was performed as detailed in 2.2.4.

5.3. Results.

5.3.1. Sucrose, Sorbitol and Water Incubations.

With 5% (w/v) sucrose, the pH fell rapidly (Table 5.1. and Fig.5.1.), and the total identifiable acid anion concentration rose concomitantly over the 24h test period (Table 5.2. and Fig.5.2.). The falls in pH (Table 5.1. and Fig.5.1.) for the 5% (w/v) sorbitol and water were slight, as were the increases in acid anion concentrations from these incubations (Table 5.2. and Fig.5.2.). These differences between the results from sucrose and both sorbitol and water were highly significant ($p<0.001$) at 24h using a Student's t-test and at the 95% Confidence Interval using Scheffé's test. There were no significant

differences between the sorbitol and water acidogenicity results.

The calcium concentration (Table 5.3.) increased with the sucrose incubation and showed little or no change with sorbitol or water. The amount of calcium released from the slabs increased daily, reflecting increasing demineralisation. Again the differences between sucrose and the sorbitol and water were highly significant ($p<0.001$) and at the 95% Confidence Interval using Scheffé's test and non-significant between sorbitol and water themselves. The high standard deviation values in the calcium analyses are the result of pooling the results over the 2 consecutive days of the experiment. The mineral profiles clearly show differences in the demineralising potential of sucrose and both sorbitol and water, and an example of each is given in Fig.5.3. (also Figs.5.4. and 5.5.).

5.3.2. Lactose, Galactose and Xylitol Incubations.

With 5% (w/v) lactose and 5% (w/v) galactose the pH fell rapidly, although not as fast or as low as the 5% (w/v) sucrose control (Table 5.4. and Fig.5.6.). The differences in pH at the end of the 24h period for both test sugars were highly significant from both sucrose and sorbitol ($p<0.001$) and at the 95% Confidence Interval using Scheffé's test but non-significant from each other. The acid anions also showed a concomitant rise as the pH fell in both cases (Table 5.5. and Fig.5.7.), and again the acid anion concentrations were significantly lower than from

Figs. 5.4. and 5.5 show colour representations of the microdensitometric profile obtained for 5% (w/v) sorbitol and 5% (w/v) sucrose respectively. The yellow line on the right hand side of the photograph is a representation of the mineral content of the e namel (the surface being at the top of the picture). The whiter the colour the more mineralised the tissue, and, conversely, the redder the picture, the more demineralised the tissue. In Fig. 5.4. no demineralisation can be seen, whereas in Fig.5.5. a small surface zone is obvious, underneath which lies the lesion body.

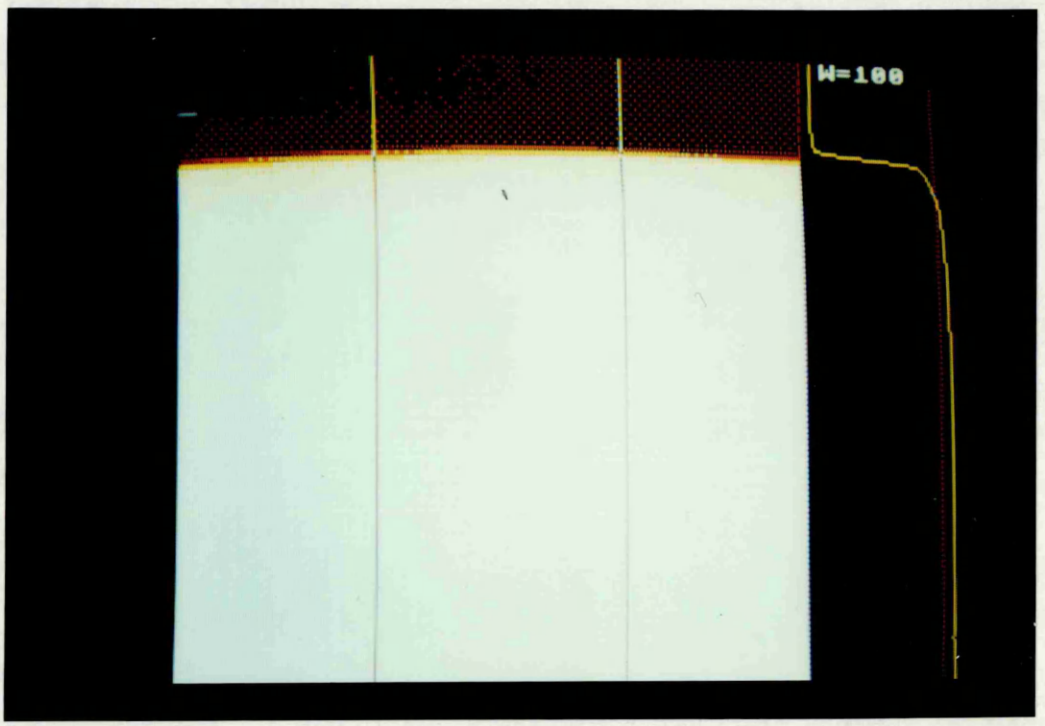

Fig.5.4. Microdensitometric Profile For 5% (w/v) Sorbitol.

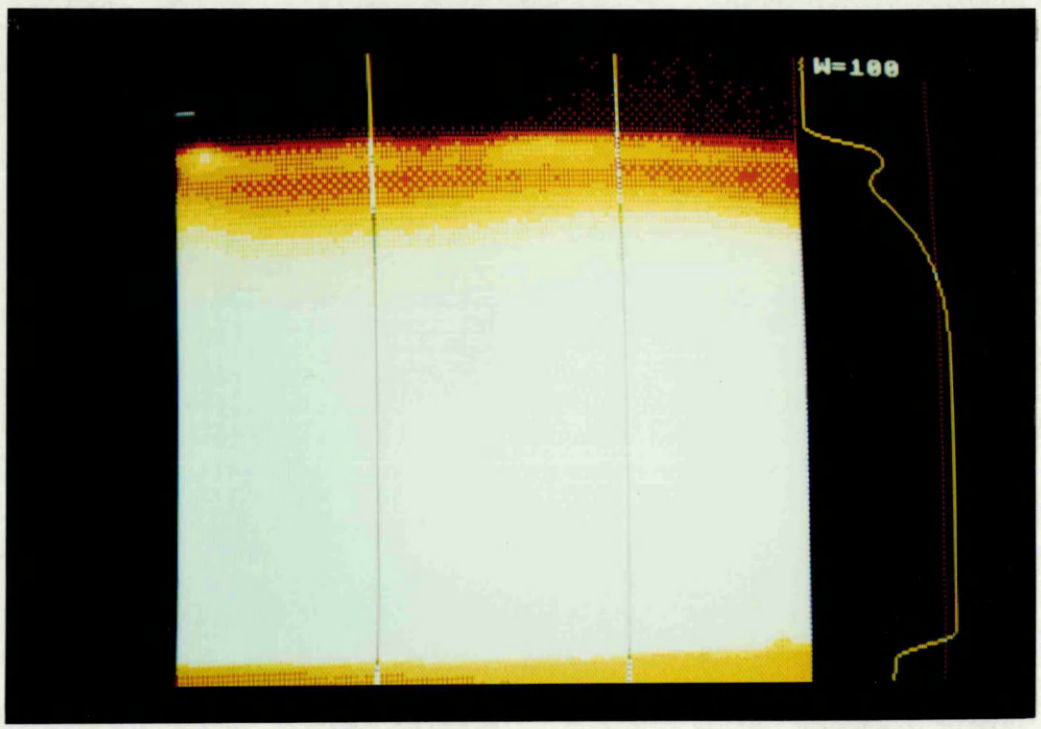

Fig.5.5. Microdensitometric Profile For 5% (w/v) Sucrose.

sucrose ($p<0.001$) and at the 95% Confidence Interval using Scheffé's test and significantly higher than from sorbitol ($p<0.001$) and at the 95% Confidence Interval using Scheffé's test. The 5% (w/v) xylitol incubation showed no marked fall in pH (Table 5.4. and Fig.5.6.) and was significantly different from sucrose ($p<0.001$) and at the 95% Confidence Interval using Scheffé's test but was not significantly different from sorbitol. The acid anion concentration showed a slight rise (Table 5.5. and Fig.5.7.) and again this was significantly different from sucrose ($p<0.001$) and at the 95% Confidence Interval using Scheffé's test and non-significant when compared to sorbitol.

The calcium results are shown in Table 5.6. and show the concentration released daily on incubation with lactose, galactose and xylitol to be significantly different from sucrose ($p<0.01$) and at the 95% Confidence Interval using Scheffé's test and from sorbitol ($p<0.05$) and at the 95% Confidence Interval using Scheffé's test.

The results from the pH, acid anion analysis and calcium assay were pooled from both days of the experiment. In the cases of pH and total acid anions there were no significant differences between the results obtained from the 1st and 2nd days. At first appearances the results for the calcium assays did look different from days 1 and 2, but on statistical analysis were not.

Table 5.1. pH at Various Time Intervals for Each Substrate (mean±SD; n=8).

Time	Sucrose	Sorbitol	Water
0 min	5.2±0.3	5.2±0.4	5.3±0.4
10min	4.7±0.2	5.0±0.2*#	5.2±0.2*#
30min	4.2±0.2	5.1±0.2*#	5.2±0.2*#
1 h	4.0±0.1	5.1±0.2*#	5.1±0.1*#
5 h	3.8±0.1	5.1±0.1*#	5.3±0.1*#
24h	4.1±0.1	5.2±0.2*#	5.5±0.2*#

*$p<0.001$ compared to the value of 5% (w/v) sucrose (Student's t-test).

significantly different from 5% (w/v) sucrose using ANOVA and thereafter at the 95% Confidence Interval using Scheffé's test.

Table 5.2. Total Acid Anions (nmol/mg wet weight bacteria) Against Time for Each Substrate (mean±SD; n=8).

Time	Sucrose	Sorbitol	Water
0 min	19.9±3.4	19.7±3.6	17.6±4.8
10min	23.2±4.5	17.2±2.4	15.4±4.8*
30min	32.6±5.1	19.6±2.9*	16.3±2.2*#
1 h	47.4±6.3	21.9±3.6*#	18.6±1.6*#
5 h	92.5±17.3	32.1±5.6*#	24.8±2.7*#
24h	163.1±19.5	47.7±6.5*#	37.5±7.1*#

* $p<0.001$ compared to the value of 5% (w/v) sucrose (Student's t-test).

significantly different from 5% (w/v) sucrose using ANOVA and thereafter at the 95% Confidence Interval using Scheffé's test.

Table 5.3. Calcium Release (mM/mm^2) for Each Substrate (mean±SD; n=8).

Time	Sucrose	Sorbitol	Water
0 min	0.1±0.1	0.1±0.1	0.2±0.2
24h	7.0±3.9	0.4±0.5*#	0.5±0.6*#

* $p<0.001$ compared to the value for 5% (w/v) sucrose (Student's t-test).

significantly different from 5% (w/v) sucrose using ANOVA and thereafter at the 95% Confidence Interval using Scheffé's test.

Table 5.4. pH at Various Time Intervals For Each Substrate (mean±SD; n=8).

Time	Sucrose	Sorbitol	Lactose
0min	4.7±0.2	4.7±0.1	4.7±0.2
10min	4.2±0.2+$	4.7±0.3*#	4.6±0.2*#
30min	3.9±0.2+$	4.5±0.2*#	4.6±0.2*#
1h	3.6±0.1+$	4.5±0.1*#	4.5±0.1*#
5h	3.8±0.1+$	4.7±0.2*#	4.6±0.2*#
24h	4.0±0.1+$	4.8±0.1*#	4.5±0.2*+#$

Time	Galactose	Xylitol
0 min	4.7±0.1	4.7±0.2
10min	4.6±0.1*#	4.6±0.1*#
30min	4.4±0.1*#	4.8±0.2*#
1 h	4.3±0.2*#	4.8±0.3*#
5 h	4.3±0.1*+#	5.1±0.5*#
24h	4.4±0.1*+#$	5.0±0.1*#

* significantly different from 5% (w/v) sucrose ($p<0.01$) using a Student's t-test

+ significantly different from 5% (w/v) sorbitol ($p<0.01$) using a Student's t-test

significantly different from 5% (w/v) sucrose using ANOVA and thereafter at the 95% Confidence Interval using Scheffé's test.

$ significantly different from 5% (w/v) sorbitol using ANOVA and thereafter at the 95% Confidence Interval using Scheffé's test.

Table 5.5. Total Acid Anions (nmol/mg wet weight bacteria) for Each Substrate (mean±SD; n=8).

Time	Sucrose	Sorbitol	Lactose
0 min	23.0±10.0	11.5±3.0	11.4±3.8
10min	26.7±8.6	14.9±4.1	12.6±3.7*#
30min	30.4±9.4+$	15.6±2.6*#	13.6±3.1*#
1h	62.0±22.2+$	20.1±4.3*#	22.0±4.4*#
5h	117.8±16.3+$	32.0±7.5*#	47.7±5.9*+#
24h	201.8±29.5+$	60.8±4.6*#	110.8±23.0*+#$

Time	Galactose	Xylitol
0 min	18.0±4.9	11.0±6.0
10min	17.7±4.5	11.8±6.0*#
30min	19.8±3.4#	14.8±6.2*#
1h	34.3±8.2*+#	19.6±8.0*#
5h	73.5±10.3*+#$	25.7±6.9*#
24h	127.9±30.1*+#$	52.3±7.8*#

* significantly different from 5% (w/v) sucrose ($p<0.01$) using a Student's t-test.

+ significantly different from 5% (w/v) sorbitol ($p<0.01$) using a Student's t-test.

significantly different from 5% (w/v) sucrose using ANOVA and thereafter at the 95% Confidence Interval using Scheffé's test.

$ significantly different from 5% (w/v) sorbitol using ANOVA and thereafter at the 95% Confidence Interval using Scheffé's test.

Table 5.6. Calcium release (mM/mm2) for Each Substrate (mean±SD; n=8).

Time	Sucrose	Sorbitol	Lactose	Galactose	Xylitol
0 h	0.0	0.0	0.0	0.0	0.0
24h	1.1±0.6+$	0.1±0.1*#	0.4±0.1*+#	0.5±0.1+#$	0.0±0.0*#

* significantly different from 5% (w/v) sucrose ($p<0.01$) using a Student's t-test

\+ significantly different from 5% (w/v) sorbitol ($p<0.01$) using a Student's t-test.

\# significantly different from 5% (w/v) sucrose using ANOVA and thereafter at the 95% Confidence Interval using Scheffé's test.

$ significantly different from 5% (w/v) sorbitol using ANOVA and thereafter at the 95% Confidence Interval using Scheffé's test.

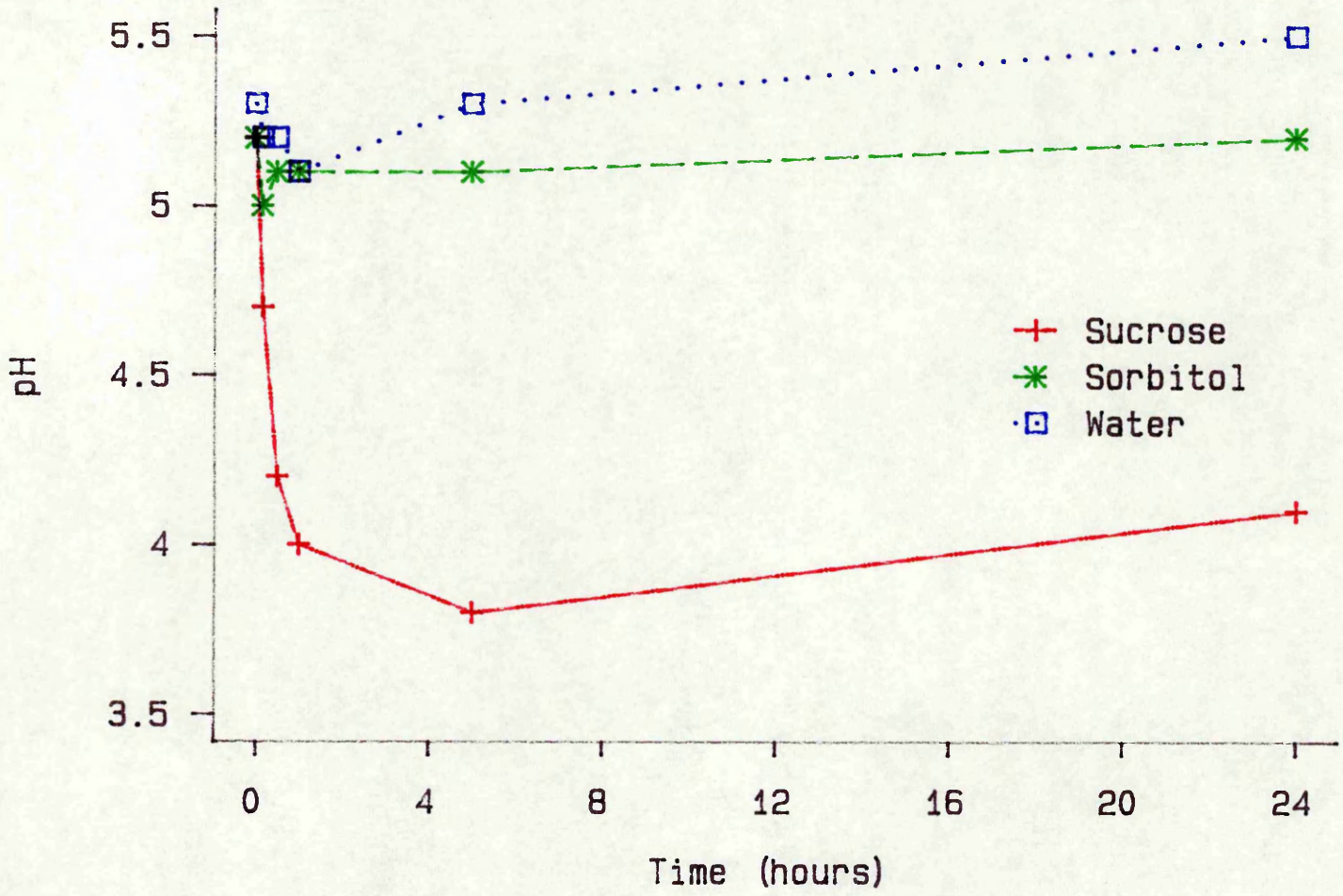

Figure 5.1. Graph of pH Against Time for Streptococcus mutans NCTC 10449 Incubated with 5% Sucrose, 5% Sorbitol and Water. (mean; n=8)

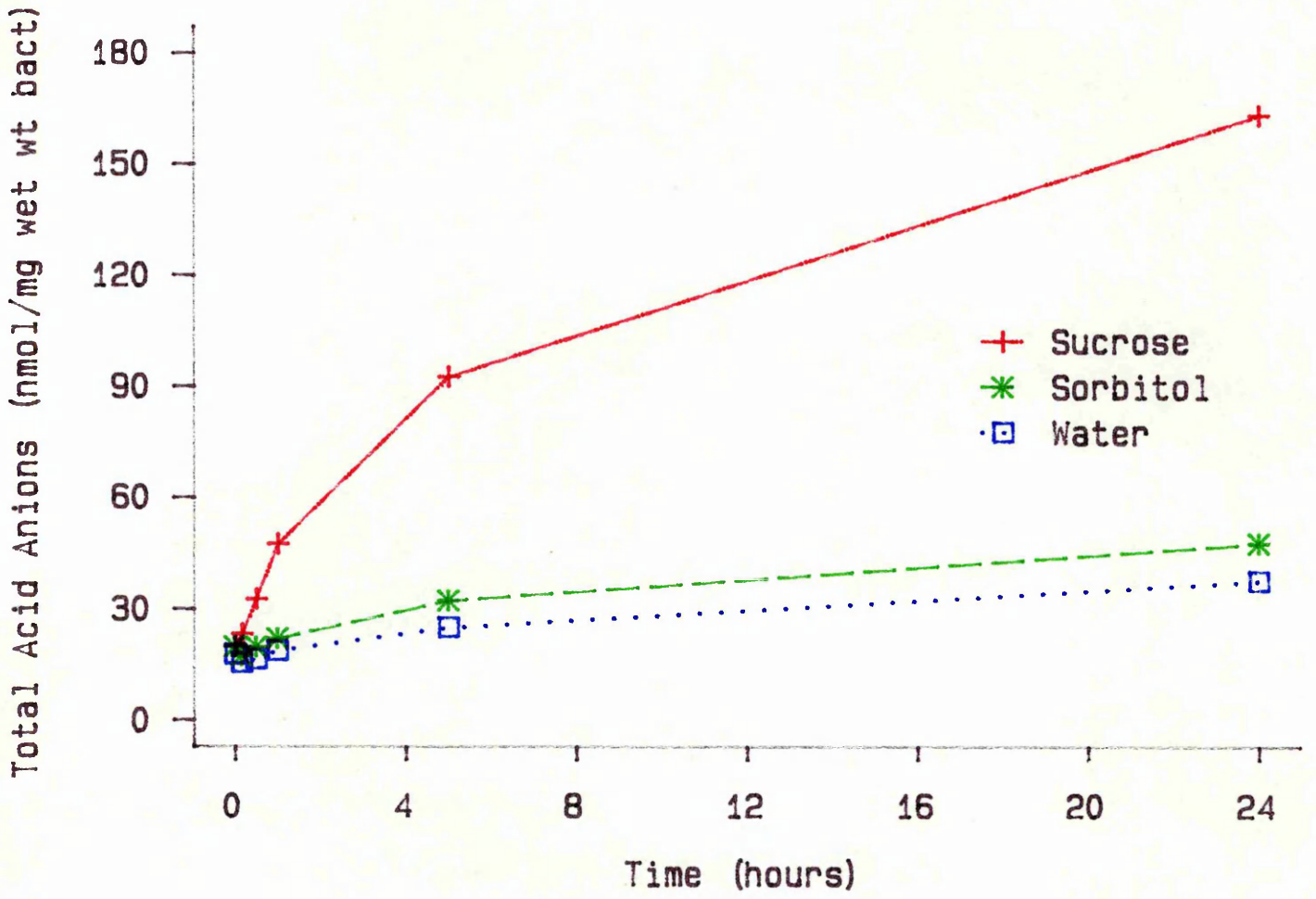

Figure 5.2. Graph of Acid Anions Against Time for Streptococcus mutans NCTC 10449 Incubated with 5% Sucrose, 5% Sorbitol and Water. (mean; n=8)

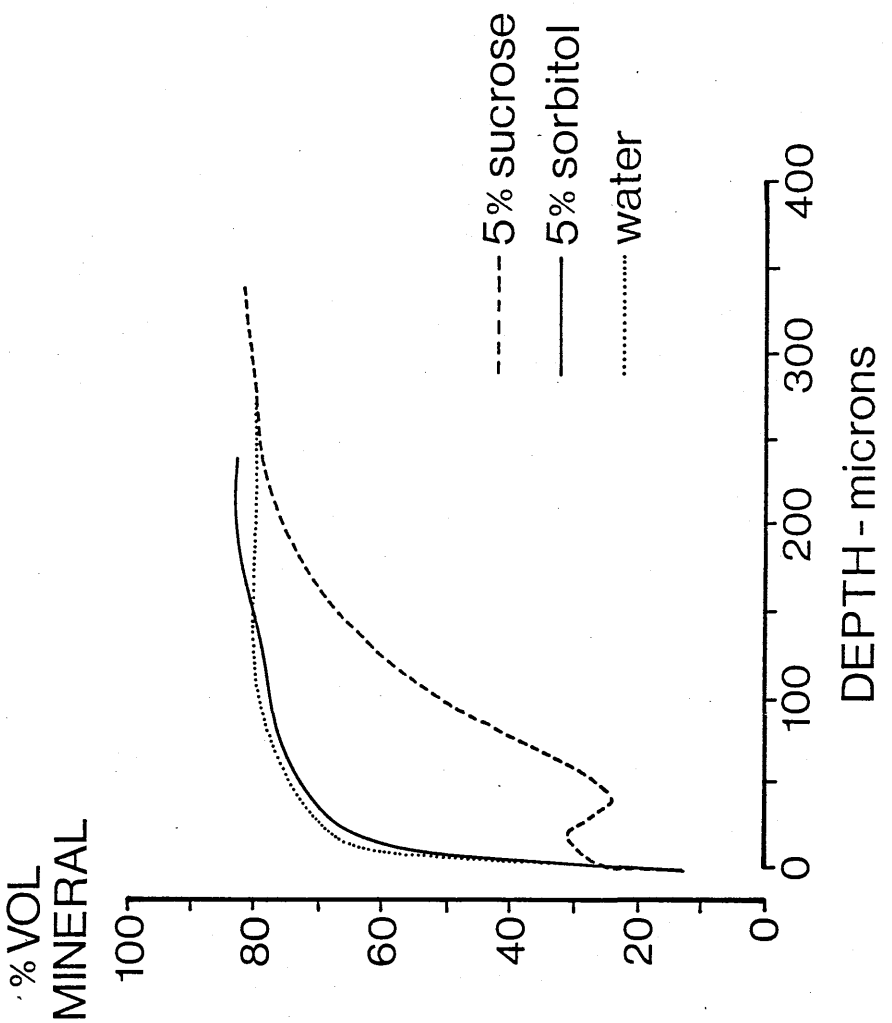

Fig. 5.3.
Apparent Mineral Content of Bovine Enamel (% by Volume)

Plotted Against Depth From Surface

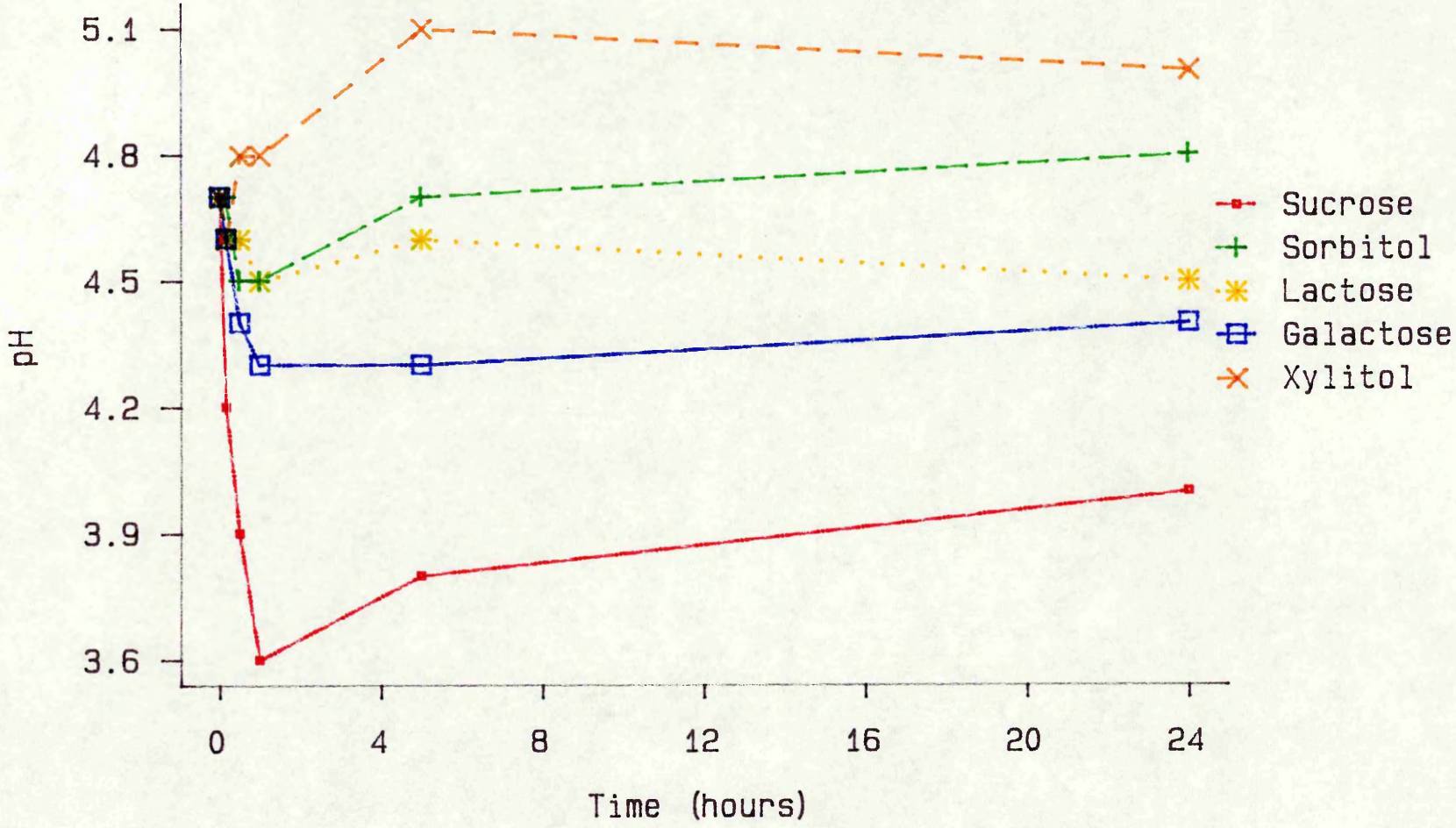

Figure 5.6. Graph of pH Against Time for 5% Solutions of Different Sugars. (mean; n=8)

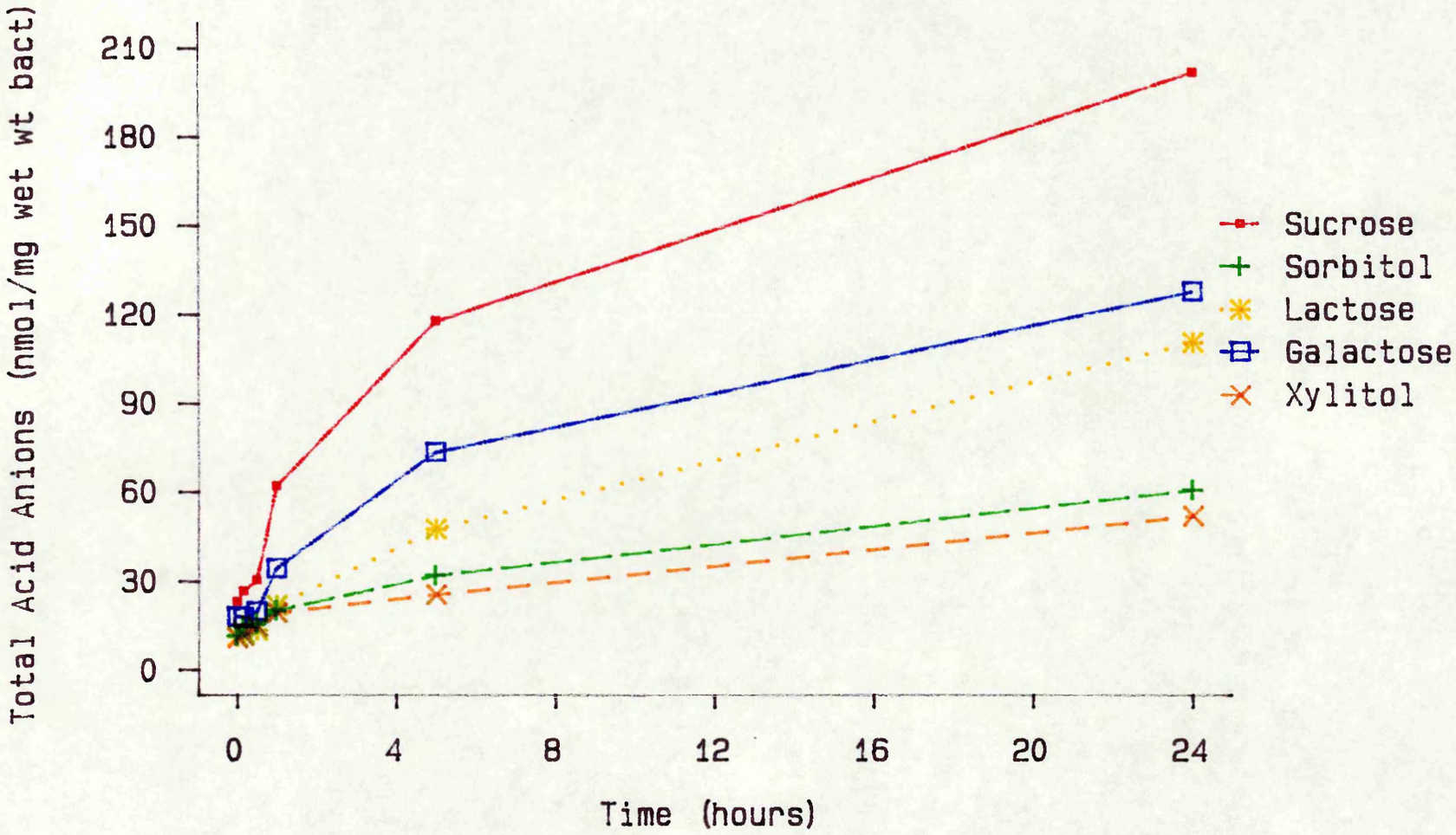

Figure 5.7. Graph of Total Identifiable Organic Acid for 5% Solutions of Different Sugars. (mean; n=8)

5.4. Discussion.

The results from the first set of experiments demonstrate that sucrose and sorbitol are valid controls in this experimental set-up, and that the system is capable of permitting measurement of the parameters of pH, acid anion concentration, and total calcium concentration, as well as being able to obtain a qualitative picture of possible lesion formation. As discussed in 4.4., for various reasons the microdensitometry cannot be used to give a quantitative result for the demineralisation using this incubation technique, but it does allow the differences among the various test substances to be seen.

The second set of experiments were carried out in order to ascertain if i) the test was capable of differentiating sugars of known differing cariogenic potential, and ii) to see how the results obtained using this test system compare with results obtained by other workers using other methods. The results for lactose, galactose and xylitol show that this test is indeed capable of differentiating solutions of different cariogenic potentials. The results indicate that the cariogenic potential of the sugar solutions is in the order, from most cariogenic to least acidogenic: sucrose >> lactose = galactose >> sorbitol > xylitol.

Other workers have documented that xylitol has a low cariogenic potential (Loesche, et al, 1984; Firestone and Navia, 1986b, and Söderling, et al, 1987). Xylitol is a five-carbon sugar alcohol which is transported into the

bacteria using a phosphoenolpyruvate:fructose phosphotransferase system and xylitol 5-phosphate is produced which builds up in the bacterial cell and eventually kills it (Reiner, 1977). However, clinical trials have shown that some bacteria can adapt to xylitol (Rateitschak-Plüss and Guggenheim, 1982). There appears to be a growth inhibition by xylitol on pure cultures of some bacteria which is not shown by other sugar alcohols, (Tuompo et al, 1983). Other sugar alcohols, such as sorbitol and mannitol are less cariogenic than sucrose as a result of their metabolic pathway. Sorbitol is a six-carbon molecule, which is transported by phosphotransferase systems into the cell and when fermented, three molecules of NADH are formed for each molecule of sorbitol. Therefore in order to preserve the oxidation-reduction balance the pyruvate formate-lyase pathway must be utilised to oxidise more than one NADH. More ethanol than acetate will be formed when the sugar alcohols are fermented and therefore their cariogenic potential is reduced. Various workers have reported that there is some adaptation by bacteria to sorbitol (Guggenheim, 1968; Havenaar, et al, 1978 and Birkhed, et al, 1978 and 1979), although it is not always clear whether this is due to the enzyme induction by the bacteria to metabolise sorbitol as a carbon source or whether it is due to selection of bacteria with the ability to utilise sorbitol. It has been found that low concentrations of glucose repress the ability of the oral bacteria to induce the necessary enzymes for sorbitol catabolism (Tatevossian

and Gould, 1976), and it has been suggested that the concentration of glucose commonly found in parotid saliva would be sufficient to do this (Slee and Tanzer, 1983).

Lactose, the disaccharide of glucose and galactose, is transported into the cell by a phosphotransferase system (Calmes, 1978) and perhaps by a permease (Hamilton and Lebtag, 1979). Workers have found that this sugar is less cariogenic than sucrose, possibly as a result of it not forming extracellular polysaccharides, but that it still presents a cariogenic challenge to the teeth (Stephan, 1940; Birkhed et al, 1981; Brudevold et al, 1983). Galactose has also been found to be less cariogenic than sucrose, and, being one of the moieties of lactose, it could be expected to have similar cariogenic properties (Brudevold et al, 1983). As this test is capable of differentiating substances of known cariogenic potential, it should therefore be possible to test foods of interest and subsequently grade them as having a cariogenic potential: greater than or equal to that of sucrose, less than or equal to that of sorbitol, or between that of sucrose and sorbitol. Foods in this last category should be considered for testing in the human plaque model and, if indicated from pH results, in the animal caries model.

CHAPTER 6

DETERMINATION OF THE CARIOGENIC POTENTIAL OF FOODSTUFFS

6.1. Introduction.

Having developed the method for assessing the cariogenic potential of solutions it was necessary to test foodstuffs of known cariogenic potential to ensure that the test system was capable of dealing with the more complex nature of food. At the same time it was possible to test unknown foodstuffs and assess their cariogenic potential. The first aim of these experiments was therefore to test foodstuffs which have been investigated by other workers using other methods and compare the results obtained with those obtained using this system, and secondly to test foodstuffs of unknown cariogenic potential.

6.2. Materials and Methods.

6.2.1. Food Processing.

The foodstuffs chosen for the 'known' cariogenic potential were i) milk chocolate[1],

ii) mint flavoured boiled sweets[2](MFS), and

iii) raw, unsalted peanuts.(Fig.6.1.)

[1] Cadbury's 'Dairy Milk' : milk, sugar, cocoa mass, cocoa butter, vegetable fat, emulsifier (E442), flavouring.

[2] 'Polo Mints', Rowntree Mackintosh : sugar, glucose syrup, stearic acid, gelatin, flavouring, antioxidant (E320).

The foodstuffs for the 'unknown' cariogenic potential were iv) Bombay Mix, and

v) Tropical Treats.(Fig.6.2.)

Fig.6.1. 'Known' Foodstuffs.

Fig.6.2. 'Unknown' Foodstuffs.

Both of these foods are readily available as mixes in health food shops and from other outlets and are sold as 'healthy' alternatives to sweets and other snack foods. Bombay Mix(BM) consists of a curried blend of peanuts, chick peas, lentils and soya crisps, and Tropical Treats(TT) is a mixture of dried bananas, pineapples, raisins, peanuts and coconuts.

The foodstuffs were treated in the following ways prior to being used in the test system:

i) The milk chocolate was weighed and double-distilled, de-ionised water added to make a 5% (w/v) mixture. The chocolate/water mixture was then placed in a water bath at 37°C until the chocolate had melted.

ii) The mint flavoured boiled sweets were weighed and double-distilled, de-ionised water added to make a 5% (w/v) mixture. The mint sweet/water mixture was then placed in a water bath at 37°C until the sweet had dissolved.

iii), iv) and v) The peanuts, Bombay Mix and Tropical Treats were ground to a powder with a mortar and pestle and then a 5% (w/v) mixture made up with double-distilled, de-ionised water and the mixtures brought to 37°C in a water bath.

6.2.2. Experimental Procedures and Analyses.

The experimental procedures and analyses were carried out as in 5.2.1. using 5% (w/v) sucrose as a positive control and 5% (w/v) sorbitol as a negative control.

6.2.3. Statistical Analysis.

The statistical analyses were performed on the results

as detailed in 2.2.4.

6.3. Results.

6.3.1. 'Known' Foodstuffs.

Table 6.1. and Fig.6.3. show the pH changes measured as a result of incubating different foodstuffs with the bacterial - enamel mixture. The pH of both the milk chocolate and the mint flavoured boiled sweets were similar in profile to that of the 5%(w/v) sucrose control, there being no significant differences between them. The pH fell rapidly on addition of the substrate and reached a minimum at 30min-1h incubation. In each case the minimum lay in the pH range 3.3-3.5. Like the sucrose control the pH fall from the chocolate and the mint flavoured sweets was significantly greater than from that of sorbitol from 30min onwards ($p<0.001$, using a Student's t-test, and at the 95% Confidence Interval using Scheffé's test). The pH of the peanuts was higher than that of the other substrates at the start of the *in vitro* incubation and, after addition to the bacteria and enamel fell rapidly to within the pH range of the others. After this initial rapid fall the pH stayed relatively high and constant for the rest of the experiment, rising slightly towards the end. The pH results were significantly different from those of the sucrose and sorbitol controls from 30min ($p<0.001$, using a Student's t-test, and at the 95% Confidence Interval using Scheffé's test).

Table 6.2. and Fig.6.4. show the total identifiable acid anions found at the various time intervals during the incubation. The chocolate and mint flavoured sweets again

show a similar trend to that of the sucrose control, there being no significant differences between them. They were all significantly greater than from sorbitol from 30min ($p<0.001$, using a Student's t-test, and at the 95% Confidence Interval using Scheffé's test). The total identifiable acid anions for the peanuts very closely followed that of the sorbitol control, there being no significant differences between them, but they were significantly less than the sucrose control from 30min ($p<0.001$, using a Student's t-test, and at the 95% Confidence Interval using Scheffé's test).

Table 6.3. shows the total calcium release by the enamel slabs during the two day incubation. The calcium release by the incubation of both the chocolate and the mint flavoured sweets was not significantly different from sucrose but significantly greater than that from sorbitol ($p<0.001$, using a Student's t-test, and at the 95% Confidence Interval using Scheffé's test). The calcium release from the peanut incubation was not significantly different from that of sorbitol but was significantly less than from that of sucrose ($p<0.001$, using a Student's t-test, and at the 95% Confidence Interval using Scheffé's test). Although microradiographic and microdensitometric analyses were carried out on these foodstuffs, it became apparent that the results obtained using these techniques were being adversely affected by the problems highlighted in Chapter 4.4. This technique has great potential as a screening tool, but the additional work required to make it suitable for use in this set-up was beyond the time and

scope of the present project. With this in mind it was decided to omit the results obtained using these methods from the remainder of this study. The procedures were carried out however, to enable further evaluations and development to be carried out at a later date.

6.3.2. 'Unknown' Foodstuffs.

Table 6.1. and Fig.6.3. show pH against Time for the incubations of BM and TT with the bacteria and enamel mixture. The BM shows an slight initial fall, lasting about 30min, in pH followed by a gradual increase. The values of pH are significantly different from both the sucrose and sorbitol controls from 30min ($p<0.001$, using a Student's t-test, and at the 95% Confidence Interval using Scheffé's test). The TT pH results show a rapid fall in pH, reaching a minimum at 30min-1h. These results showed no significant difference from those of the sucrose control, but were significantly lower than from the sorbitol control from 30min ($p<0.001$, using a Student's t-test, and at the 95% Confidence Interval using Scheffé's test).

Table 6.2. and Fig.6.4. show the total identifiable acid anion results for the foodstuffs. The acid anion profile for BM is very similar to that of the sorbitol control, there being no significant differences. The results are, however, significantly lower than from sucrose from 30min ($p<0.001$, using a Student's t-test, and at the 95% Confidence Interval using Scheffé's test). The TT results show no significant differences from the results

obtained from the sucrose control, but show significantly more acid ($p<0.001$, using a Student's t-test, and at the 95% Confidence Interval using Scheffé's test) than from 30min from the sorbitol control.

Table 6.3. shows the total calcium release during the daily incubations. The results from BM were significantly lower than from the sucrose control ($p<0.001$, using a Student's t-test, and at the 95% Confidence Interval using Scheffé's test), but showed no significant differences from the sorbitol control. The TT showed no significant differences from the sucrose but was significantly greater ($p<0.001$, using a Student's t-test, and at the 95% Confidence Interval using Scheffé's test) than from the sorbitol control.

Table 6.1. pH at Various Time Intervals for Each Substrate (mean±SD; n=8).

Time	Sucrose	Sorbitol	Chocolate
0 min	4.2±0.2	4.3±0.2	4.5±0.4
10min	3.8±0.2$	4.3±0.2#	4.0±0.2
30min	3.5±0.2+$	4.2±0.2*#	3.5±0.2+$
1 h	3.4±0.2+$	4.2±0.1*#	3.3±0.2+$
5 h	3.7±0.1+$	4.4±0.1*#	3.6±0.1+$
24h	4.1±0.1+$	4.7±0.1*#	3.9±0.1+$

Time	MFS	Peanuts	BM	TT
0 min	4.3±0.2	5.1±0.2#$	4.8±0.2#	4.7±0.2
10min	3.8±0.2	4.3±0.2#	4.3±0.2#	4.2±0.2
30min	3.5±0.2+$	4.0±0.1*+#	4.0±0.1*+#	3.6±0.1+$
1 h	3.4±0.2+$	4.0±0.1*+#$	4.0±0.1*+#	3.4±0.1+$
5 h	3.6±0.1+$	4.3±0.1*+#$	4.3±0.1*+#	3.6±0.1+$
24h	3.9±0.1+$	4.4±0.1*+$	4.4±0.1*+#$	3.9±0.1+#$

* significantly different from 5% (w/v) sucrose $p<0.001$ using a Student's t-test.

+ significantly different from 5% (w/v) sorbitol $p<0.001$ using a Student's t-test.

significantly different from 5% (w/v) sucrose using ANOVA and at the 95% Confidence Interval using Scheffé's test.

$ significantly different from 5% (w/v) sorbitol using ANOVA and at the 95% Confidence Interval using Scheffé's test.

Table 6.2. Total Acid Anions(nmol/mg wet weight bacteria) Against Time for Each Substrates (mean±SD; n=8).

Time	Sucrose	Sorbitol	Chocolate
0 min	26.4±9.5	16.5±5.5	12.7±3.0
10min	41.5±20.8$	23.0±8.9#	25.9±4.8
30min	56.8±11.2+$	24.9±3.7*#	59.4±21.0+$
1h	91.8±10.8+$	29.7±4.0*#	89.4±22.8+$
5h	162.9±55.8+$	41.7±5.1*#	161.4±25.2+$
24h	224.4±79.3+$	58.4±8.4*#	201.5±56.9+$

Time	MFS	Peanuts	BM	TT
0 min	15.1±6.1	9.9±6.2	3.2±5.0#$	5.5±5.6
10min	26.2±6.4	20.4±6.8#	10.4±4.1#	16.3±8.2#
30min	51.1±10.3+$	31.0±11.1*#	21.2±5.5*#	39.1±12.3+
1h	85.6±12.2+$	39.4±13.6*#	25.7±6.8*#	68.8±23.8+$
5h	134.1±13.2+$	45.6±12.3*#	34.1±6.9*#	125.9±23.1+$
24h	178.6±29.5+$	60.4±8.3*#	45.0±11.7*#	157.8±41.1+$

* significantly different from 5% (w/v) sucrose $p<0.001$ using a Student's t-test.

+ significantly different from 5% (w/v) sorbitol $p<0.001$ using a Student's t-test.

significantly different from 5% (w/v) sucrose using ANOVA and at the 95% Confidence Interval using Scheffé's test.

$ significantly different from 5% (w/v) sorbitol using ANOVA and at the 95% Confidence Interval using Scheffé's test.

Table 6.3. Total Calcium Release(mM/mm^2) for Each Substrate (mean±SD; n=8).

	0h	24h
Sucrose	0.0±0.0	1.8±0.4+$
Sorbitol	0.0±0.0	0.3±0.3*#
Chocolate	0.0±0.0	1.4±0.3+$
MFS	0.0±0.0	1.5±0.3+$
Peanuts	0.0±0.0	0.4±0.1*#
BM	0.0±0.0	0.3±0.1*#
TT	0.0±0.0	1.3±0.3+$

* significantly different from 5% (w/v) sucrose $p<0.001$ using a Student's t-test.

+ significantly different from 5% (w /v) sorbitol $p<0.001$ using a Student's t-test.

significantly different from 5% (w/v) sucrose using ANOVA and at the 95% Confidence Interval using Scheffé's test.

$ significantly different from 5% (w/v) sorbitol using ANOVA and at the 95% Confidence Interval using Scheffé's test.

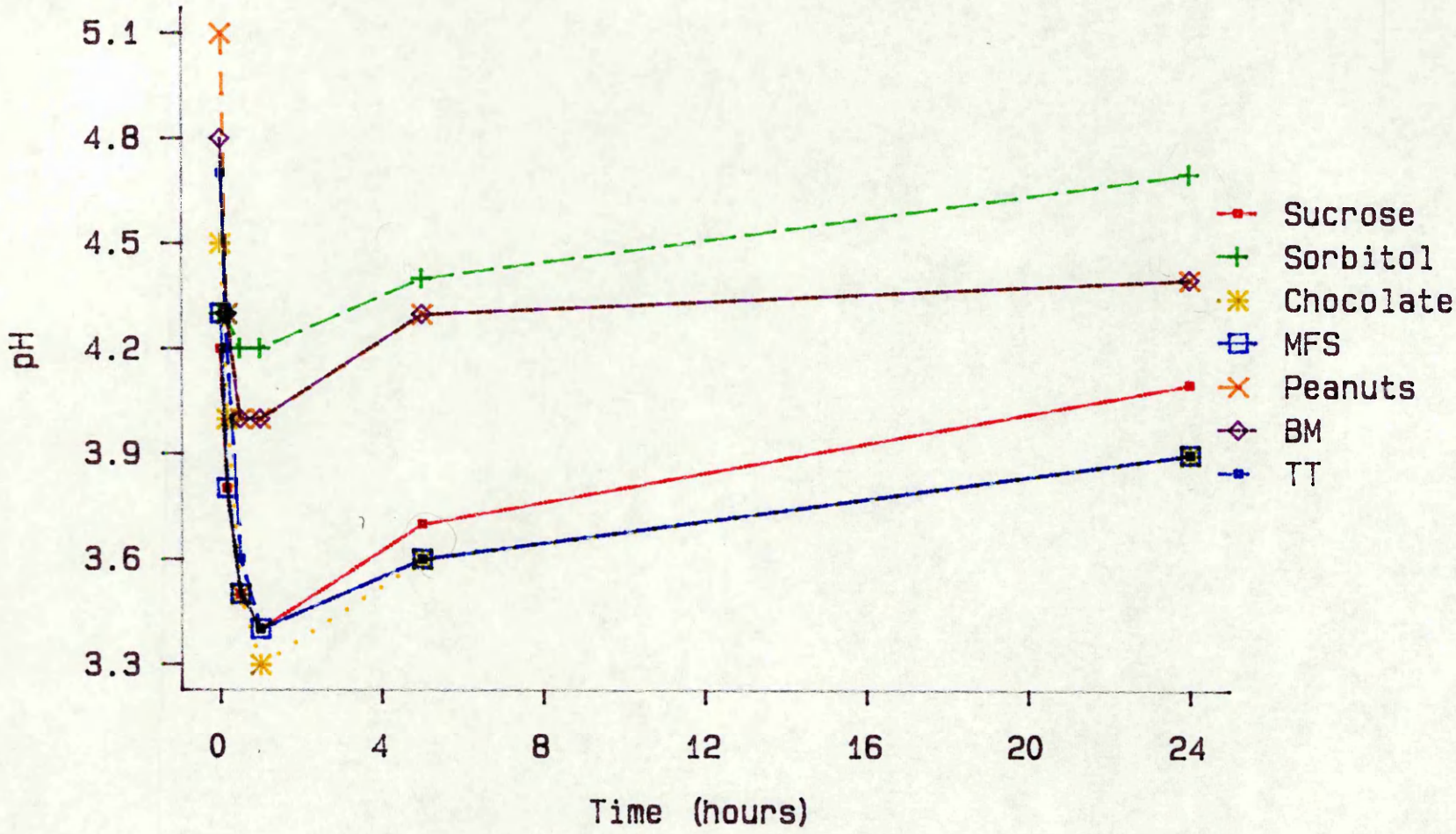

Figure 6.3. Graph of pH Against Time for 5% Mixtures of Different Foodstuffs. (mean; n=8)

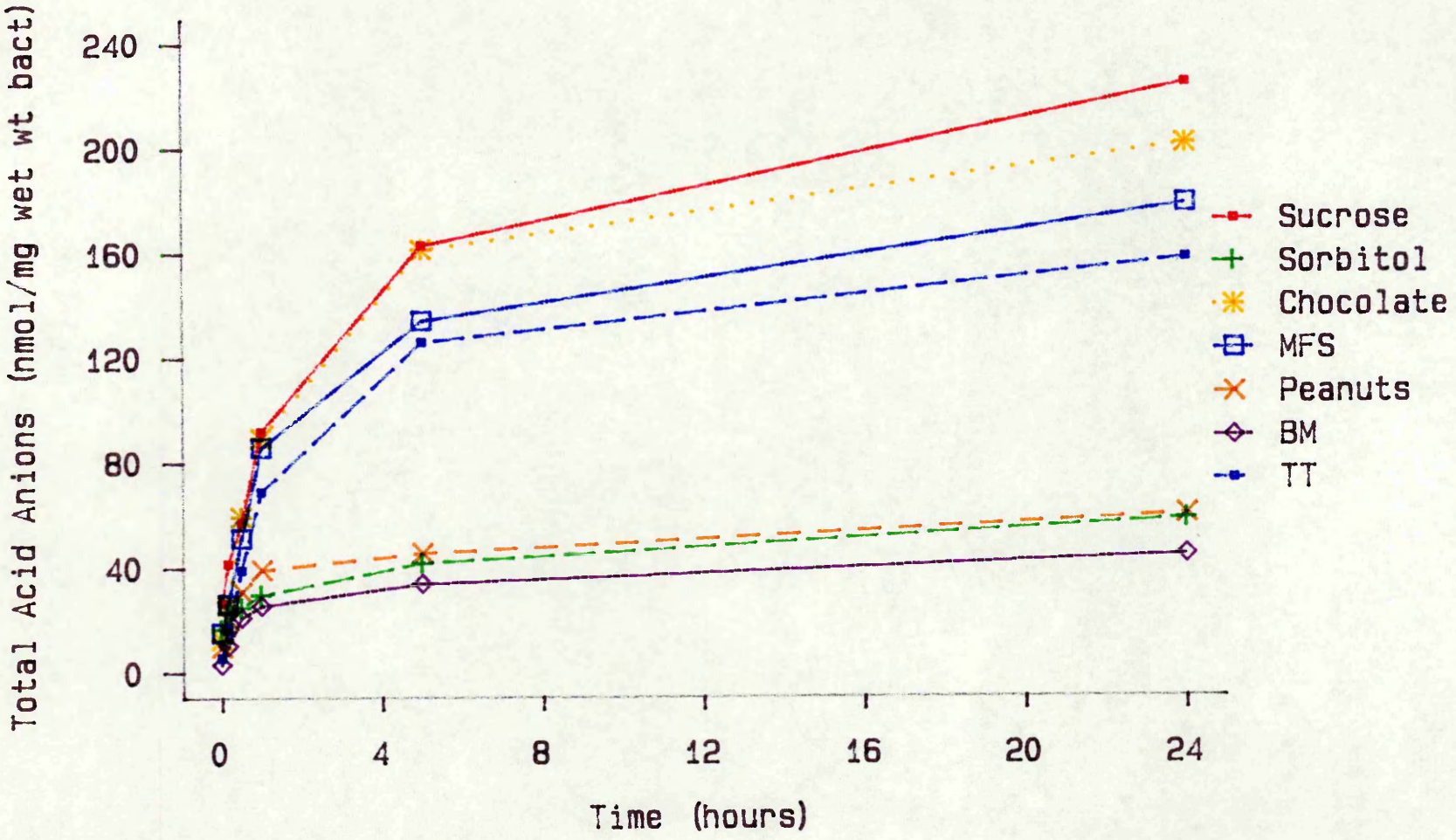

Figure 6.4. Graph of Total Identifiable Organic Acid Anions for 5% Mixtures of Different Foodstuffs. (mean; n=8)

6.4. Discussion.

The results from Tables 6.1., 6.2. and 6.3. allow the 'known' foodstuffs to be ranked, from highest cariogenic potential to lowest, in the following order : Sucrose = Mint Flavoured Sweets = Milk Chocolate >> Peanuts = Sorbitol. Other workers have used all or some of these 'known' foods in other models, and in general our findings are consistent with those found elsewhere. Edgar, et al, (1975), using acidogenicity measurements from pooled plaque found the foods to be in the order, from highest acidogenicity to lowest, Mint Flavoured Sweets > Milk Chocolate > Peanuts. Harper, et al, (1985), using both a rat programmed feeding model and plaque pH test found milk chocolate had greater cariogenic potential than peanuts. This result was also found by Rugg-Gunn, et al, (1978), using pH minimum recordings, and Mundorff and Curzon, (1985), using a programmed rat feeding model. Stephan, (1966), found using a rat feeding model, that milk chocolate had a greater cariogenic potential than did mint flavoured sweets. One set of results does not correspond to these findings however, Bibby, (1975), using bovine enamel incubated with 180 different 20% food-saliva mixtures found that the rank order was peanuts > milk chocolate. He explains his results by discussing food retention, titratable acid, enamel protective factors in food and sugar concentration. He concluded that enamel dissolution was dependent on the amount of acid contained within the food and that foods with a high sugar concentration did not destroy as much enamel as did the

foods with a lower concentration of sugar but in conjunction with starch. Saliva was used in the model of Bibby, et al, (1951), and this is not present in the model being tested here. A reason for the much greater cariogenic potential of peanuts found in Bibby's model could therefore be due to the starch in the peanuts being hydrolysed to glucose and maltose by the α-amylase in the saliva. This would produce more fermentable carbohydrate for the bacteria, and therefore more enamel dissolution. To test this hypothesis, α-amylase, in the concentration range found in saliva, would have to be added to the incubation mixture. This is investigated in Chapter 7.

There is a great deal of discussion in the literature concerning protective or modifying factors in food. Strålfors (1966), suggested that cocoa contained enamel protective factors, which led to a reduction in caries score when fed to hamsters, and in 1967a, claimed the same for chocolate. He found that the effect was not due to the fat present in the substances and, in another (1967b) paper, showed that constituents did reduce caries score (0.2% theobromine, 0.2% xanthine, 0.05% vanillin and 0.001-0.005% tannic acid). Jenkins (1970), found that aqueous extracts of cocoa reduced the solubility of the enamel, but that the minimal reduction in acid production observed was due to the buffering capacity of the extract. That chocolate has a lower cariogenic potential than might have been expected from its sugar content, has been noticed by other workers. Edgar, et al, (1975) suggested that the high sugar concentration inhibited acid production and

Rugg-Gunn, et al, (1978), Bowen et al (1980), Morrissey et al (1984) and Mundorff and Curzon (1985) all found that chocolate, although by no means non-cariogenic, has a lower than expected result. Kashket et al, (1985), found that cocoa powder extracts inhibit streptococcal glucosyltransferase, thus limiting the ability of the bacterium to metabolise glucose and sucrose. Jenkins and Smales (1966), found that various seed hulls possessed water-soluble constituents which reduced the rate of enamel solubility. As the peanuts used in these experiments were raw, ie. with their hulls, this could have had a protective effect. The method described in this thesis could be used to investigate the possible protective factors in foodstuffs if more work was carried out on it.

The results from 6.3.1., clearly show that this test is capable of distinguishing foodstuffs of high cariogenic potential and low cariogenic potential. Milk chocolate and the mint flavoured sweets had a cariogenic potential in the same order as that of the 5% (w/v) sucrose control. The peanuts had a cariogenic potential in the same order as that of the sorbitol control. In a screening system, therefore, it would be necessary to further test the milk chocolate and the mint flavoured sweets in animal caries/human plaque pH models before making a conclusive decision as to their cariogenic potential. In the case of peanuts, as the results showed that the peanuts had a cariogenic potential less than or equal to that of the 5% (w/v) sorbitol control, further testing can be deemed unnecessary and the food declared to be of low cariogenic

potential. However, with regard to the α-amylase discussed above, this conclusion concerning peanuts cannot be drawn until experiments using α-amylase have been carried out (Chapter 7).

In the case of the 'unknowns', the foodstuffs can be ranked from highest cariogenic potential to lowest:

Sucrose = TT >> BM = Sorbitol

From these results it would be concluded that, as TT has a cariogenic potential equal to that of the sucrose control, further testing should be carried out using <u>in vivo</u> human plaque pH and/or rat caries models. As BM has a cariogenic potential equal to that of the sorbitol control no further testing should be required. Again, like the peanuts, BM contains starch which could be hydrolysed into fermentable carbohydrates on the action of α-amylase. This conclusion cannot therefore be drawn until the necessary checks have been made (Chapter 7),

CHAPTER 7

FURTHER EXPERIMENTS TO INVESTIGATE THE CARIOGENIC POTENTIAL OF FOODSTUFFS BY A) ADDITION OF SALIVARY AMYLASE AND B) *in vivo* HUMAN PLAQUE pH STUDY

7.1. Introduction.

As discussed in Chapter 6, a problem highlighted in the food testing experiments was the possible under-evaluation of a food's cariogenic potential due to the absence of salivary amylase. In the *in vivo* situation the salivary amylase present in the mouth would hydrolyse starchy components of the food into maltose (Mörmann and MÜhlemann, 1981), and so give the oral micro-organisms access to these sugars. Without the presence of this enzyme, the test system could be giving starchy foods an unrealistically low cariogenic potential. The aim of these experiments was to look at this problem in two ways i) add salivary amylase to the test system, in the concentrations normally found *in vivo*, and see what effect, if any, this had on the cariogenic potential of the food, and ii) to carry out *in vivo* human plaque pH studies using the foodstuffs and compare the results obtained using the test system with those found using this more thorough model.

7.2. Materials and Methods.

7.2.1. Addition of Salivary Amylase.

The experimental procedures and analyses were carried out as described in 5.2.1. using 5% (w/v) sucrose as a positive control and 5% (w/v) sorbitol as a negative

control, with salivary amylase (Type 1X-A, Sigma Chemical Company Ltd., Poole, Dorset, England.) being added to the incubation mixtures in the final concentration of 0.38g/l (Mason and Chisholm, 1975) when required. Samples were only taken at 0h and 24h for analysis by isotachophoresis but the pH was taken as before. The exception to the analyses was that of the calcium, as due to the presence of sodium citrate in the amylase preparation, the colorimetric assay could not be employed. The calcium was therefore measured by the method of Robinson et al (1971). 0.3ml of test solution was mixed in a 1cm cell with 0.3ml of freshly prepared ice-cold 5mg% solution of calcein in a 0.75M KOH. A blank, consisting of 0.3ml water and 0.3ml calcein, was read against the test solution at 506nm. Extinction values were compared to those obtained from 0.3ml aliquots of calibration solutions containing 2.0, 2.5, 3.0 and 3.5μg calcium/ml. These calcium estimations were carried out by Mr. D.A. Weetman. The substances tested were i) 5% (w/v) sucrose, ii) 5% (w/v) sucrose + amylase, iii) 5% (w/v) sorbitol, iv) 5% sorbitol (w/v) + amylase, v) 5% (w/v) starch (uncooked corn starch, Sigma Chemical Company Ltd., Poole, Dorset, England.), iv) 5% (w/v) starch + amylase, vii) 5% (w/v) peanuts, viii) 5% (w/v) peanuts + amylase, ix) 5% (w/v) BM, x) 5% BM (w/v) + amylase, xi) 5% (w/v) TT and xii) 5% (w/v) TT + amylase.

7.2.2. In vivo Human Plaque pH Study.

Five adult, dentate volunteers were asked to refrain from all forms of oral hygiene for 24h prior to the

experiment. They were also asked to avoid eating or drinking anything except water for 2h before the experiment so that their plaque would be 'resting' (Geddes, 1974) and were asked to note the content and time of the last thing consumed before the experiment, as a check that the subjects had complied with the instructions given. They were each asked to rinse their mouths with a 10% (w/v) sucrose solution for 1 min, and plaque samples taken from all available smooth surfaces avoiding restorations at 0, 3, 6, 8,and 10min. The time taken for plaque sampling was as close as possible to one minute. One minute after sampling, the plaque pH was measured using a Beckman 'one-drop' electrode (Beckman,-R11C Ltd., Glenrothes, Fife, Scotland)(Geddes and M^{a}^{c}Fadyen, 1980) and the subjects checked to ensure that their plaque pH fell to 5.5 or below following rinsing. One subject did not pass this criterion and was therefore excluded from the remainder of the experiment (Scientific Consensus Conference on Methods for the Assessment of the Cariogenic Potential of Foods, 1986). The other four subjects were asked to repeat the previous regime of abstaining from all forms of oral hygiene for 48h prior to the experiment and from food or drink for 2h before the experiment. They then either:

i) rinsed for 1 min with 10% (w/v) sucrose (positive control), or

ii) rinsed for 1 min with 10% (w/v) sorbitol (negative control), or

iii) ate 'normal' portion of BM (the eating time was measured and time zero of the Stephan curve taken as the

time at the end of eating), or

iv) ate 'normal' portion of TT (the eating time was measured as above).

The plaque pH was measured at 0, 3, 6, 8, 10, 15 and 30min and the area from the 'resting pH' to the actual pH was calculated. The experiments were repeated at the same time each day to minimise the effect of circadian rhythms on saliva production.

7.2.3. Statistical Analysis.

The statistical analyses were performed on the results as detailed in 2.2.4.

7.3. Results.

7.3.1. Addition of Salivary Amylase.

Fig. 7.1. and Table 7.1. show the pH record during the experiment. The results for sucrose, sorbitol and TT show that at all times there are significant differences (using a Student's t-test), at various significance levels between the substrate with and without amylase. TT and TT with amylase are significantly different at the 95% Confidence Interval using Scheffé's test after 10min, as are sucrose and sucrose with amylase at 30min and 1h, and sorbitol with and without amylase at 5h. Starch and peanuts both show non-significant differences at 0 and 10min, but thereafter all the times show significant differences at varying significance levels (using a Student's t-test). Using ANOVA and Scheffé's test, starch and starch with amylase (starch having a significantly higher pH than starch+amylase) are significantly different at 5h, and peanuts and peanuts (peanuts being significantly higher than peanuts+amylase) with amylase are significantly different after 30min, BM

shows non-significant differences at 0, 1, 5 and 24h and significant differences at 10min ($p<0.05$) and 30min ($p<0.01$) (using a Student's t-test), and shows no significant differences from the amylase containing mixture using ANOVA.

Table 7.2. shows the total acid anion production during the test. There are non-significant differences between all the substrates with and without the addition of salivary amylase except in the cases of starch, where there (starch significantly lower than starch+amylase) is a significant difference at 24h $\wedge$ ($p<0.02$, using a Student's t-test) and at the 95% Confidence Interval using (peanuts significantly lower than peanuts+amylase) Scheffé's test; peanuts, $\wedge$ ($p<0.05$, using a Student's t-test), and BM, ($p<0.01$, using a Student's t-test).

Table 7.3. shows the total calcium results over the experiment. The results between the substrate with and without the salivary amylase are non-significant (using a Student's t-test), with the exception of sorbitol, peanuts and BM. The differences between the starch with amylase and the BM with amylase are significant at $p<0.001$ and the difference between the sorbitol and sorbitol plus amylase are significant at $p<0.01$. Sucrose and sucrose with amylase, and sorbitol and sorbitol with amylase, are significantly different throughout the test using Scheffé's test.

7.3.2. In vivo Human Plaque pH Study.

Fig. 7.2. and Tables 7.4. and 7.5. show the results obtained from this preliminary human plaque pH study. The (from resting to) area $\wedge$ the curve ranks the foodstuffs in the order:

Sucrose = TT >> BM = Sorbitol. This result is consistent with the findings of the developed *in vitro* demineralisation model with and without inclusion of salivary amylase. (Chapter 6.) The area $\wedge$ from resting to the curve has been shown by Edgar (1976), to follow a Gaussian distribution, and therefore parametric statistical analysis may be used. The area $\wedge$ from resting to the curve for sucrose was significantly greater than that of BM ($p<0.01$, using a Student's t-test), and at the 95% Confidence Interval using Scheffé's test, but not significantly different from TT. The area $\wedge$ from resting to the curve for sorbitol was significantly lower than from TT ($p<0.01$, using a Student's t-test) but not significantly different from BM.

Table 7.1. pH at Various Time Intervals for Each Substrate (mean±SD; n=6). (α-a = α-amylase)

Time	Sucrose	Suc+α-a	Sorbitol	Sor+α-a
0 min	4.2±0.2	4.7±0.1[▫]	4.3±0.2	4.6±0.1[^]
10min	3.8±0.2	4.2±0.1[ø]	4.3±0.2	4.6±0.1[^]
30min	3.5±0.2	3.9±0.1[ø#]	4.2±0.2	4.5±0.1[^]
1 h	3.4±0.2	3.7±0.1[^#]	4.2±0.1	4.5±0.1[▫#]
5 h	3.7±0.1	3.9±0.1[^]	4.4±0.1	4.7±0.1[▫]
24h	4.1±0.1	4.3±0.1[^]	4.7±0.1	5.0±0.1[▫]
Time	Starch	Sta+α-a	Peanuts	Pean+α-a
0 min	4.6±0.1	4.7±0.1	5.1±0.3	5.1±0.2
10min	4.6±0.1	4.6±0.1	4.5±0.2	4.7±0.2
30min	4.6±0.1	4.4±0.1[^]	3.8±0.2	4.4±0.1[▫#]
1 h	4.6±0.1	4.4±0.1[^]	3.9±0.2	4.3±0.1[ø#]
5 h	4.8±0.1	4.4±0.1[▫#]	4.2±0.1	4.5±0.1[▫#]
24h	4.9±0.2	4.6±0.1[^]	4.3±0.1	4.7±0.2[ø#]

[▫] significantly different from the non-amylase containing substrate with $p<0.001$, using a Student's t-test.

[^] significantly different from the non-amylase containing substrate with $p<0.01$, using a Student's t-test.

[+] significantly different from the non-amylase containing substrate with $p<0.05$, using a Student's t-test.

[ø] significantly different from the non-amylase containing substrate with $p<0.002$, using a Student's t-test.

[#] significantly different from the non-amylase containing substrate using ANOVA, and at the 95% Confidence Interval using Scheffé's test.

Table 7.1.(continued) pH at Various Time Intervals for Each Substrate (mean±SD; n=6). (α-a = α-amylase)

Time	BM	BM+α-a	TT	TT+α-a
0 min	4.8±0.2	5.0±0.2	4.7±0.2	5.1±0.2ˆ
10min	4.3±0.2	4.6±0.2+	4.2±0.2	4.7±0.1ø#
30min	4.0±0.1	4.2±0.1ˆ	3.6±0.1	4.1±0.1ø#
1 h	4.0±0.1	4.1±0.1	3.4±0.1	3.8±0.1ø#
5 h	4.3±0.1	4.3±0.1	3.6±0.1	3.9±0.1ø#
24h	4.4±0.1	4.3±0.1	3.9±0.1	4.2±0.1ø#

ø significantly different from the non-amylase containing substrate with $p<0.001$, using a Student's t-test.

ˆ significantly different from the non-amylase containing substrate with $p<0.01$, using a Student's t-test.

+ significantly different from the non-amylase containing substrate with $p<0.05$, using a Student's t-test.

ø significantly different from the non-amylase containing substrate with $p<0.002$, using a Student's t-test.

significantly different from the non-amylase containing substrate using ANOVA, and at the 95% Confidence Interval using Scheffé's test.

Table 7.2. Total Acid Anions (nmol/mg wet weight bacteria) Against Time for Different Substrates (mean±SD; n=6). (α-a = α-amylase)

Substrate	0 h	24h
Sucrose	26.4±9.5	224.4 ±79.3
Suc+α-a	33.4±23.4	310.3 ±60.7
Sorbitol	16.5 ±5.5	58.4 ±8.4
Sor+α-a	17.4±12.0	71.9 ±16.3
Starch	16.0 ±6.0	76.9 ±46.8
Sta+α-a	19.2±12.9	243.4±127.2*#
Peanuts	9.9 ±6.2	60.4 ±8.3
Pean+α-a	15.2±17.3	109.5 ±51.5+
BM	3.2 ±5.1	45.0 ±11.7
BM+α-a	19.8±24.0	157.8 ±69.7^
TT	5.5 ±5.6	157.8 ±41.1
TT+α-a	17.1±16.9	176.1 ±33.8

* significantly different from the non-amylase containing substrate with $p<0.02$, using a Student's t-test.

+ significantly different from the non-amylase containing substrate with $p<0.05$, using a Student's t-test.

^ significantly different from the non-amylase containing substrate with $p<0.01$, using a Student's t-test.

significantly different from the non-amylase containing substrate using ANOVA, and at the 95% Confidence Interval using Scheffé's test.

Table 7.3. Total Calcium Release (mM/mm2) Against Time for Different Substrates (mean±SD; n=6). (α-a = α-amylase)

Substrate	0h (SD)	24h(SD)
Sucrose	0.0±0.0	2.9±1.0
Suc+α-a	1.3±0.6##	3.5±1.3##
Sorbitol	0.0±0.0	0.3±0.1
Sor+α-a	1.3±0.8##	0.9±0.4^##
Starch	0.1±0.1	0.3±0.2
Sta+α-a	0.6±0.3	2.0±0.9°°
Peanuts	0.0±0.0	0.4±0.1
Pean+α-a	1.0±0.4	0.3±0.1
BM	0.0±0.0	0.3±0.1
BM+α-a	1.3±0.6	1.9±1.0°°
TT	0.0±0.0	1.8±0.5
TT+α-a	1.7±1.1	2.4±1.0

^ significantly different from the non-amylase containing substrate with $p<0.01$, using a Student's t-test.

°° significantly different from the non-amylase containing substrate with $p<0.001$, using a Student's t-test.

significantly different from the non-amylase containing substrate using ANOVA and at the 95% Confidence Interval using Scheffé's test.

Table 7.4. Plaque pH Against Time After Consumption of Various Substrates (mean±SD; n=4).

Time	Sucrose	Sorbitol	BM	TT
0 min	7.3±0.3	7.2±0.2	7.4±0.5	7.1±0.1
3 min	5.5±0.4	7.2±0.3	7.0±0.5	6.3±0.6
6 min	5.6±0.4	7.3±0.3	7.4±0.5	6.2±0.7
8 min	5.9±0.8	7.4±0.4	7.3±0.6	6.2±0.5
10min	6.2±0.5	7.4±0.4	7.4±0.5	6.5±0.6
15min	6.2±0.5	7.4±0.4	7.3±0.4	6.7±0.2
30min	6.6±0.3	7.3±0.3	7.2±0.2	6.9±0.2

Table 7.5. Area $\wedge$ From Resting to the Stephan Curve After Consumption of Various Substrates (mean±SD; n=4).

Substrate	Area $\wedge$ From Resting to the Curve
Sucrose	184.5 ±12.7$^{\$}$
Sorbitol	219.7 ±8.5$^{\#}$
BM	218.4 ±9.2$^{*\#}$
TT	198.3 ±6.8^{+}

* significantly different from sucrose $p<0.01$ using a Student's t-test.

+ significantly different from sorbitol $p<0.01$ using a Student's t-test.

significantly different from sucrose using ANOVA and at the 95% Confidence Interval using Scheffé's test.

$ significantly different from sorbitol using ANOVA and at the 95% Confidence Interval using Scheffé's test.

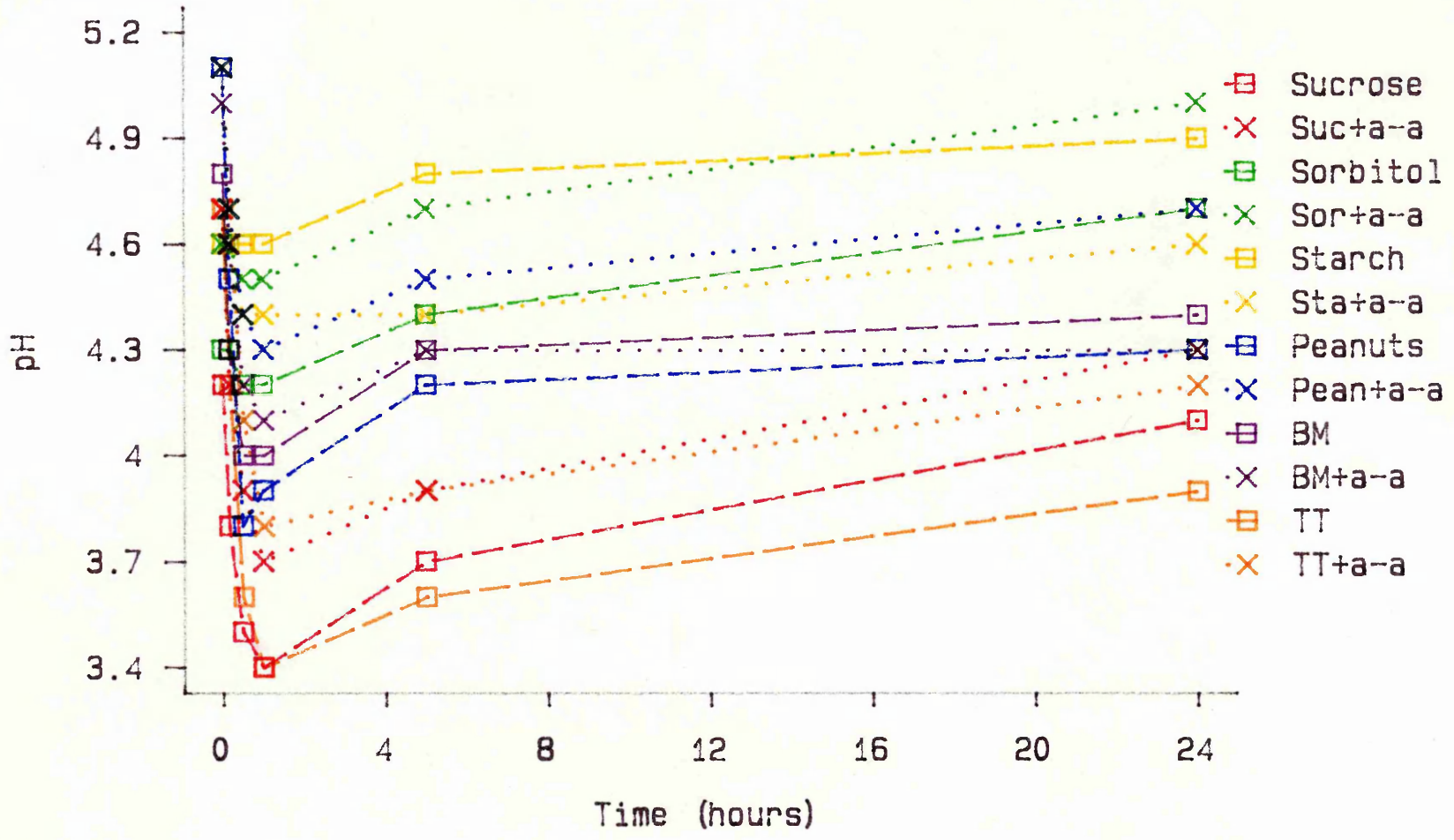

Figure 7.1. Graph of pH Against Time for Different Dietary Carbohydrates With and Without Salivary Amylase. (mean; n=8)

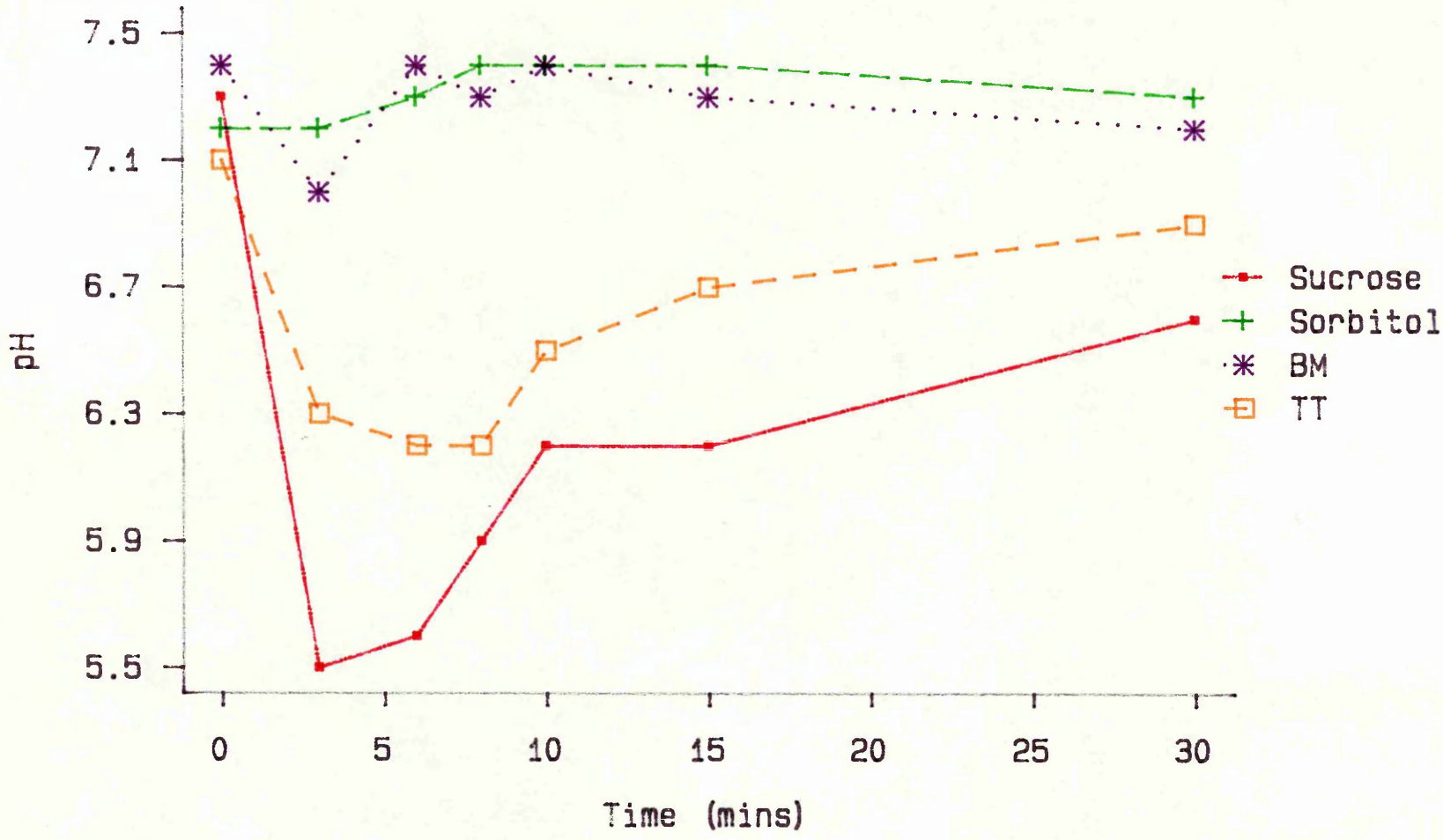

Figure 7.2. Graph of pH Against Time for Different Dietary Carbohydrates in vivo. (mean; n=4)

7.4. Discussion.

7.4.1. Addition of Salivary Amylase.

The results from the in vitro demineralisation model with the addition of salivary amylase are difficult to explain. The expected result was that the cariogenic potential of sucrose, sorbitol and perhaps TT would not change with the addition of amylase, whereas the cariogenic potential of starch, peanuts and BM would increase with its addition. However, as can be seen from the results, the pattern does not emerge as clearly as that. The acid anion results fit closely to the predicted results, but the pH and the calcium release results do not. In our standard model, the pH results give exactly the same pattern as the total acid anion concentration, however, in the salivary amylase obtained from the chemical company, ammonium sulphate and sodium citrate were used as "balance agents" for the amylase. These chemicals could be acting as buffers on the system, thus absorbing the H^+ formed during the fermentation, and masking the true effects on the hydrogen ion formation. More work would obviously be required to overcome this problem, the buffering capacity of the system could be checked when the amylase is added, and perhaps the salivary amylase could be obtained without the presence of these two buffering compounds. The addition of saliva itself could be considered, although this adds so many more variables to the system, and it is not possible to obtain a regular supply of saliva of constant composition, so that any change on the cariogenic potential of the foods found

as a result could not be attributed to the effects of the salivary amylase alone. The calcium results show that the addition of salivary amylase did increase the calcium loss from the enamel in the cases of sorbitol, starch and BM. The latter two could be expected, but the increased loss in the case of sorbitol is strange. It could also have been expected that the peanuts would have shown an increase in mineral loss, which they did not appear to. As discussed in Chapter 6, the peanut hulls appear to exert a protective effect on the enamel, which is perhaps due to (a) substance(s) in the hull which affected the solubility of the enamel (Jenkins and Smales, 1966), and this might be the effect we are seeing here. Another problem with the addition of salivary amylase to the system is that it would have an unrepresentative amount of time to act on the starch, if any were present. In vivo the action of the salivary amylase is limited by the period of time the food is in the mouth and the amount of time in the stomach until the hydrochloric acid in the stomach penetrates the food bolus and denatures the enzyme. Using this model, the enzyme can act throughout the 2X24h cycles of the experiment and therefore can exert a much more profound effect than that which would be seen in vivo , apart from the action of food trapped in interproximal areas or in fissures. These problems make it difficult to assess the contribution of salivary amylase to the system, and more work would be needed to clarify the situation. This would be advisable, as the presence of starch in various foodstuffs could compromise the appropriateness of the in

vitro demineralisation model if this answer is not known.

7.4.2. *In vivo* Human Plaque pH Study.

The results from the *in vivo* human plaque pH study confirm the results found by the *in vitro* demineralisation model as obtained in Chapter 6. This test was carried out to check that there were no major differences between the results obtained from the developed model and those obtained using plaque pH measurement, and further work with greater numbers of subjects is to be done. The 'Scientific Consensus Conference on Methods For the Assessment of the Cariogenic Potential Of Foods' (1986), recommended the use of *in vivo* human plaque pH measurement as a reliable test for the cariogenic potential of foods, and also recommended that a food found by an *in vitro* demineralisation model to be of cariogenic potential between that of sucrose and sorbitol should be further tested in one of human plaque pH and animal caries models. The results from this test would indicate that the BM is of low cariogenic potential, whereas the TT has a relatively high cariogenic potential, and therefore should not be recommended as a 'safe' snack.

CHAPTER 8

CONCLUSIONS

The initial purpose of the research described in this thesis was to refine an existing method for studying plaque - substrate - enamel interactions, for use as an initial screening test in the estimation of the cariogenic potential of snack foods. Health care professionals need the necessary information about foodstuffs to give informed advice to their patients, and with increased public awareness of healthy eating they can now provide their patients with informed advice to enable them to adopt a healthier diet. Shortly after this project was started, there was a conference held in San Antonio, Texas, which was attended by many of the main workers in the field of cariogenicity testing. Their aim was to discuss the current methodologies employed to study this subject, and, if possible, to establish set guidelines for the different methods. This would allow researchers to be assured that they were working to a standardised format and their results could be directly comparable to those of others working in similar fields. This standardisation would also allow maximum information to be gained from the experimental results published in this field of research. The delegates of the conference suggested the need for an initial screening test for foodstuffs to be developed, so that foods could be evaluated as to their appropriateness for testing in more exhaustive and time-consuming and costly methods, and so maximise the use of resources. A method which did not require expert personnel to carry it

out would also be important, if people in less developed countries or with limited resources were to use it to screen indigenous foodstuffs. With this in mind, the aim of the thesis was changed slightly to include the criteria laid down by the conference delegates in the development of an *in vitro* screening test for foodstuffs, and modelled on the method of Geddes *et al*, 1984. The method was to be quick, cheap and simple, and should be able to give a food, by the end of the test, a rating of having a cariogenic potential of i) Greater or equal to that of 5% sucrose, ii) Less or equal to that of 5% sorbitol, or iii) Between that of 5% sucrose and 5% sorbitol. It would be foods in category iii) which would then have to be tested in the more extensive models of animal caries models or human plaque pH models.

The model of Geddes *et al*, is a 5 day recycling demineralisation model, in which human tooth slabs are incubated with pooled human plaque and a test substance. The plaque and substrate are changed daily, and the pH, acid anions and Knoop hardness measurements on the enamel recorded. The delegates at San Antonio had recommended that a homogeneous bacterial slurry of *Streptococcus mutans* be used as an artificial plaque and that either abraded human or bovine enamel employed. Chapters 3 and 4 of this thesis, detail the developments of this system to fulfil the criteria laid down by the conference delegates. The developed system then required to be tested to ensure that it was able to discriminate first solutions and then foods of different cariogenic potential. The initial experiments

were done with sucrose, sorbitol and water to ensure that the positive and negative controls were adequately discriminated. The next step was to test different sugar solutions of known cariogenic potential and ensure that these too could be adequately discriminated. Subsequent to this, foodstuffs, already tested by other workers in other test systems were screened, and the ability of the method to deal with the more complex nature of foods determined. After these criteria had been fulfilled, foodstuffs of unknown cariogenic potential were then put into the system and a rating obtained as to their cariogenic potential. Salivary amylase was also added to the developed system to see what effect, if any, this enzyme made to the results obtained because some of the test foods contained cooked starch. To check these results, the same foods were tested *in vivo* in a human plaque pH experiment and the results obtained with this more comprehensive test compared with those of the new *in vitro* model.

The developed system can be summarised thus:
Abraded, bovine tooth slabs were incubated with a 5% (w/v) mixture of test foodstuff and double-distilled, de-ionised water and a slurry of *Streptococcus mutans*, grown to early stationary phase in a glucose containing, chemically defined medium. The bacteria to substrate ratio was 3μg/ml, and the mixture was incubated in an aerobic, orbital incubator for 24h before the enamel slab was washed and replaced into a vial containing fresh bacteria and substrate. The pH was measured at intervals throughout the

course of the experiment, and samples taken for acid anion and total calcium analysis. The enamel slabs were then examined for lesion formation and the applicability of the techniques of microradiography and microdensitometry for the enamel slabs used in this system evaluated. All the tests were run with the inclusion of a 5% (w/v) solution of sucrose as a positive control and 5% (w/v) sorbitol solution as a negative control.

There are the following drawbacks to this method: apart from the inclusion of salivary amylase when the test foods contained starch, no account is taken of the salivary influence, neither the buffering effect nor the volume of saliva released and no attempt has been made to mimic the retention times of the foods in the mouth, all the test substances are left to interact with the enamel for 24h although in most sites in the human mouth this length of time would be unrepresentative. There is only a brief remineralisation phase in the test system (when the slab is placed in the fresh incubation mixture every 24 hours), to allow the maximum cariogenic challenge to the enamel and thus the test errs on the side of overestimating a food's cariogenic potential. This test does not aim to give a definitive statement about the safety of the food with respect to its cariogenicity, but only seeks to maximise the available resources by excluding the foods for which there is clearly no need to continue with the cariogenic potential testing programmes, as discussed in Chapter 1 (1.8). Further work needs to be done to allow the potentially very useful techniques of microradiography and

microdensitometry to be used at the end of this experimental procedure, and some further appraisal of the need for inclusion of salivary amylase into the system is also required.

In the course of development of this *in vitro* model, it has become apparent that, the analytical procedures could be cut-down, so that fewer pH and acid analyses need be carried out for each test run. The calcium measurement and pH measurements taken in concert appear to be a very sensitive indicator of a food's cariogenic potential and it is possible that a very rapid pre-screen could be developed using these parameters alone. When testing foods for the first time in this system however, it would be prudent to include all the parameters to ensure that there were no differences from previous tests.

Other workers have used a similar approach to investigate different facets of the caries process. Dummer *et al*, (1982), used human teeth with enamel windows and a culture of *Streptococcus mutans* NCTC 10832, in a sequential batch culture technique. Previous workers had found that without remineralisation steps in an acid attack, caries-like lesions did not occur, and only enamel etching took place. This group found that lesions comparable in form to natural lesions could be created when the teeth were placed in fresh bacterial cultures daily for periods of 7 to 26 days. Clarkson *et al*, (1984), again used human teeth, which were placed in a bacterial gel of *Streptococcus mutans* FA1. The teeth were replaced in a fresh bacterial gel every 48h and then removed and a longitudinal section

taken after two weeks and then weekly for a total of six weeks. By this means the progress of caries lesions could be assessed. This group also found that they could produce lesions consistent with the form normally found *in vivo*. In a subsequent study (1987), these workers examined the ability of *Streptococcus mutans* Ingbritt and *Actinomyces viscosus* Be32 to form carious lesions in the presence of 0.2% sucrose, 0.1% sucrose and 0.1% starch, 0.1% sucrose + 0.1% starch and α-amylase and 0.2% starch and α-amylase. They found that *Streptococcus mutans* was unable to form carious lesions when the sucrose concentration was limited and that its ability to form lesions using starch as a substrate was amylase dependent. *Actinomyces viscosus* was not limited by these constraints. These two models showed that it was possible to produce lesions of the *in vivo* pattern in a 'simple' *in vitro* system, and that it was also possible to examine carbohydrates in this type of test.

The method which has been developed which most closely follows the method described in this thesis is that of Pearce and Hampton (1987). They developed an *in vitro* fermentation model to test the cariogenicity of foods. Their model was different from this model in the following respects: only the components of the food likely to be able to diffuse into plaque were fermented; the subsaturation or supersaturation of the plaque fluid was assessed; there was no negative control; enamel was not used but a fluorhydroxyapatite containing 1000ppm F^-. Two different simulated oral fluids were formulated. They both contained Ca, P, F, Na, K and Mg but fluid 1 also contained lactate,

acetate and propionate and fluid 2 contained albumin and various amino acids. Both fluids had a pH of 6.5, and were designed to have a 'critical pH' of 5.5. Streptococcus mutans AHT was grown for 18h in Todd Hewitt broth and the centrifuged pellet was washed and resuspended in simulated oral fluid at a concentration of 5mg dry weight organism / 500μl. This mixture was added to test food dialysates, in the ratio 50μl / ml, which had been obtained by placing the food slurry in either a dialysis chamber or a dialysis sac immersed in simulated oral fluid, undisturbed for 24h at 5°C. The pH of the fermentation mixture was recorded, as were the inorganic phosphate and fluoride concentrations. Samples were also analysed for lactate, acetate and propionate. The foods tested were sweetened yoghurt; vanilla wine biscuit; milk chocolate; mild cheese; strawberry sweetened milk and pasturised whole milk. The results showed that the cariogenic potential of the foods ranked them in the order, from highest cariogenic potential to lowest : biscuit > strawberry milk > sweetened yoghurt > milk chocolate > whole milk > cheese. These results are largely in agreement with those found by other workers, although chocolate comes out with a higher cariogenic potential than found in rat caries models.

The main objective of this project was to develop an in vitro test system which would allow the rapid analysis of a foodstuff's cariogenic potential, and to assess the need for that foodstuff to undergo more rigourous testing in more time - consuming and expensive systems. This would hopefully minimise cost and time, and would free

researchers to concentrate their energies and resources on the foods whose cariogenic potentials are more debatable rather than having to expend needless time and energy on all foods. In completing this thesis, I hope that my work will prove useful to other workers in this field, and that this technique will be used.

APPENDIX 1

THEORY OF ISOTACHOPHORESIS

The meaning for the term 'isotachophoresis' may be found by looking at the two Greek words from which it is derived, that of 'iso' meaning same and 'tacho' meaning speed. Thus isotachophoresis is a technique whereby the different ionic species are sandwiched between a leading electrolyte of greater mobility than any of the species under scrutiny and a terminating electrolyte of mobility less than any of the species being studied. Once the ionic species within the system have separated themselves according to charge, they are then constrained to travel at the same speed, and thus the species are lined up according to ionic charge within the capillary, the length of each species block being directly related to the quantity present in the original sample.

APPENDIX 2

METHOD OF ISOTACHOPHORESIS TRACE ANALYSIS

A 5mmol/l standard solution of formic, pyruvic, phosphoric, lactic, succinic, acetic and propionic acids was made up, and standard calibration curves for each acid obtained by injecting 0.5μl to 3μmol concentrations of the acids into the column, obtaining the zone lengths and then plotting them against the known nanomolar concentration injected (see Fig. 1). The intercept and the gradient were then obtained from the subsequent regression analysis and used in calculating the unknown concentrations from the experimental zone lengths.

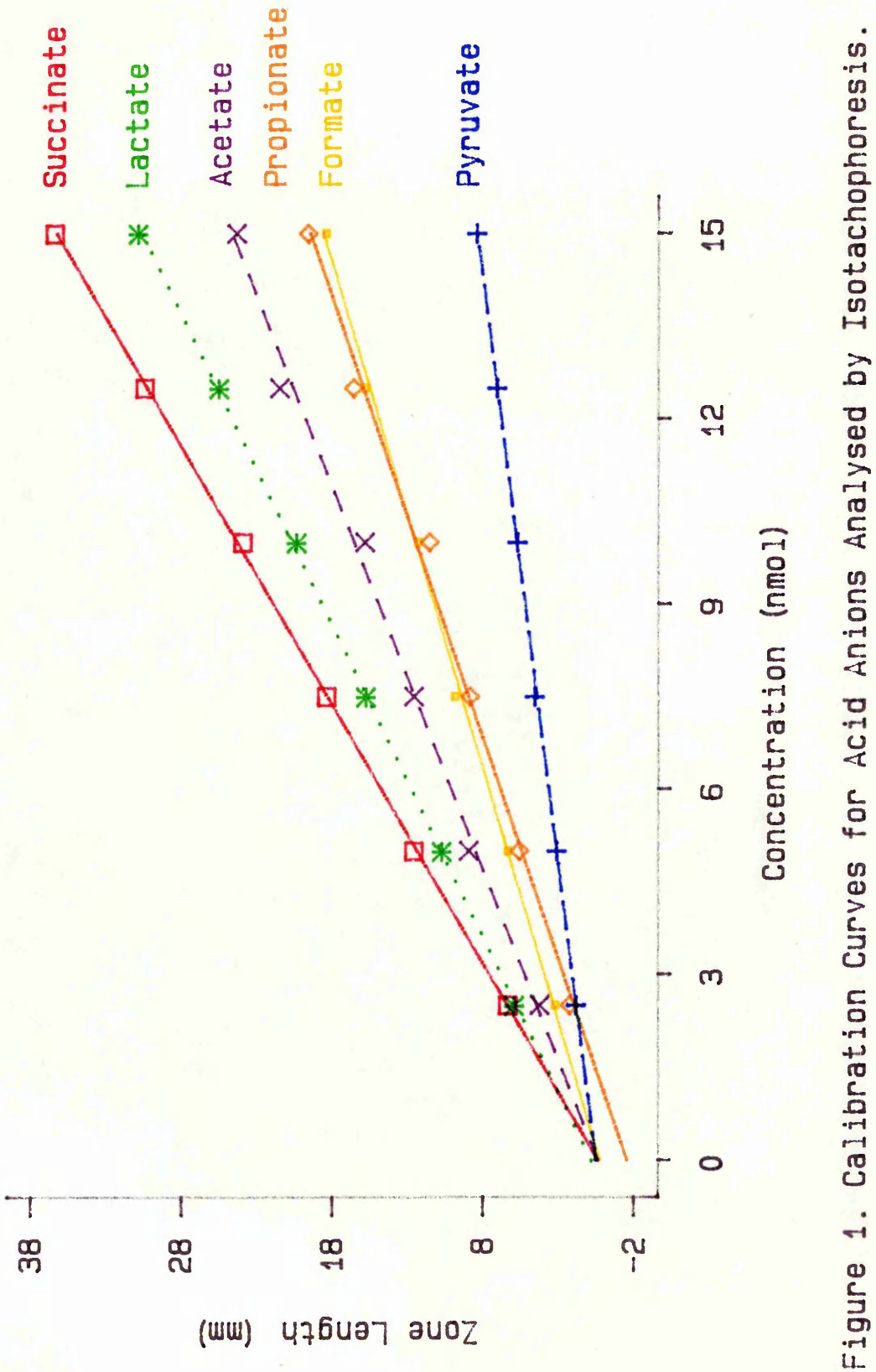

Figure 1. Calibration Curves for Acid Anions Analysed by Isotachophoresis.

APPENDIX 3

api 20 STREP DIAGNOSTIC SYSTEM

'The api 20 STREP diagnostic system consists of 20 microtubes containing dehydrated substrates for the demonstration of enzymatic activity or the fermentation of sugars. A dense suspension is made from a pure culture and used to rehydrate the enzymatic substrates and the metabolic end products produced during the incubation period are either revealed through spontaneous coloured reactions or by the addition of reagents. The fermentation tests are inoculated with an enriched medium which reconstitutes the sugar substrates and the fermentation of carbohydrates is detected by a shift in the pH indicator.

The tests are as follows:

TEST	SUBSTRATES	REACTIONS/ENZYMES
VP	Pyruvate	Acetoin Production
HIP	Hippurate	Hydrolysis
ESC	Esculin	β-glucosidase
PYRA	Pyrrolidonyl 2 naphthylamide	Pyrrolidonylarylamidase
αGAL	6-Bromo-2-naphthyl α-D-galactopyranoside	α-galactosidase
βGUR	Naphthol AS-B1 β-D-glucuronate	β-glucuronidase

βGAL	2-naphthyl-β-D galactopyranoside	β-galactosidase
PAL	2-naphthyl phosphate	Alkaline Phosphatase
LAP	L-leucine-2-naphthyl amide	Leucine arylamidase
ADH	Arginine	Arginine dihydrolase
RIB	Ribose	Acidification
ARA	L-Arabinose	Acidification
MAN	Mannitol	Acidification
SOR	Sorbitol	Acidification
LAC	Lactose	Acidification
TRE	Trehalose	Acidification
INU	Inulin	Acidification
RAF	Raffinose	Acidification
AMD	Starch(2)	Acidification
GLYG	Glycogen	Acidification

The test is carried out as follows:

Place a test strip into an incubation box to which about 5ml of distilled water has been added to create a humid environment. Using a sterile swab, harvest all the culture from a previously prepared subculture plate and make a dense suspension. Distribute the suspension with a sterile pipette in the first half of the strip. Open an ampoule of 'api 20 STREP medium' and transfer the remaining suspension into it and homogenize. Distribute this into the remaining wells. Cover tests ADH to GLYG with mineral oil. Close the incubation box and incubate at 35 - 37°C for 4 hours to obtain a first reading and 24 hours to obtain a second reading. After 4 hours add the reagents:

VP test - 1 drop of VP1 and VP2.

HIP test - 2 drops of NIN.

PYRA, αGAL, βGUR, βGAL, PAL, LAP tests - 1 drop of ZYM A and ZYM B. Wait 10 minutes, read the reactions according to the colour chart. Reincubate for the remaining 20 hours and reread the reactions ESC, ADH, RIB to GLYG. Identification can then be made using the identification table supplied with the test.

The reagents used are:

api 20 STREP medium : 0.50g cystine, 20g tryptone, 5g sodium chloride, 0.50g sodium sulphite, 0.17g phenol red, 1000ml sterile water. Final pH 7.8.

NIN reagent : 7g ninhydrin, 100ml 2-methoxy ethanol.

VP 1 : 40g potassium hydroxide, 100ml distilled water.

VP 2 : 6g alpha naphthol, 100ml ethyl alcohol.

ZYM A : 25g tris-hydroxymethyl-amino-methane, 11ml hydrochloric acid, 10g lauryl sulphate, 100ml distilled water.

ZYM B : 0.35g fast blue, 100ml 2-methoxy-ethanol.

APPENDIX 4

CHEMICALLY DEFINED MEDIUM

The chemically defined medium used throughout these experiments was the FMC medium described by Terleckyj, Willett and Shockman (1975). This medium contained the following constituents per ml:

20mg D-glucose, 6mg sodium acetate, 600μg $(NH_4)_2SO_4$, 35μg adenine, 27μg guanine, 30μg uracil, 200μg $MgSO_4$, 10μg NaCl, 10μg $FeSO_4$, 10μg $MnSO_4$, 5μg glutamine, 300μg L-glutamic acid, 110μg L-lysine, 100μg each of L-aspartate, L-isoleucine, L-leucine, L-methionine, L-serine, L-phenylalanine, L-threonine and L-valine, 200μg each of DL-alanine, L-arginine, L-cystine, L-histidine, glycine, L-hydroxyproline, L-proline, L-tryptophan and L-tyrosine, 440μg KH_2PO_4, 300μg K_2HPO_4, 3.15mg Na_2HPO_4, 2.05mg NaH_2PO_4, 225μg sodium citrate, 0.4μg riboflavin, 0.01μg biotin, 0.1μg folic acid, 0.8μg pantothenate, 0.1μg *p*-aminobenzoic acid, 0.4μg thiamine, 2.0μg nicotinamide and 0.8μg pyridoxamine.

These chemicals were all analytical grade, obtained from Sigma Chemical Company (Sigma Chemical Company Ltd., Poole, Dorset, England.). The medium was made up with double distilled, de-ionised water and the pH adjusted to 6.5 ± 0.1 with NaOH. The medium was then filter sterilised with a Millipore 0.45μm filter (Millipore (U.K.) Ltd., Watford, Hertfordshire, England.), 20 ml aliquots poured aseptically into sterile glass bottles and stored at 4°C.

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Development of a Screening Test for the Determination of the Cariogenic Potential of Foods

(Short Communication)

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In cariogenicity testing two main types of tests are used: human in vivo plaque pH tests and animal caries experiments. The former provide information concerning the acidogenic potential of a substance in humans, and the latter give information concerning the cariogenic potential in an animal model. Both tests, however, can be expensive and time-consuming and have attendant problems of obtaining suitable volunteers or husbanding animals. To optimize the available resources, it would be of obvious benefit to have a rapid, inexpensive test which would allow the substance of interest to be investigated and would identify foodstuffs of particular interest prior to further testing in the above-mentioned tests. The aim of obtaining information about the cariogenic potential of foodstuffs is to enable clear positive dietary advice to be given to the public.

The Scientific Consensus Conference on Methods for Assessment of the Cariogenic Potential of Foods [1986] made several recommendations for this type of test: a homogeneous slurry of *Streptococcus mutans*, type c, should be used as an 'artificial plaque'; abraded bovine enamel should be used and positive and negative controls employed, for which sucrose and sorbitol, respectively, were suggested.

The aim of the present study was to modify the 5-day recycling in vitro demineralization model of Geddes et al. [1984] with the above recommendations in mind and to obtain a screening test which would give a rapid indication of a foodstuff's cariogenic potential.

S. mutans 10449 was grown to early stationary phase in a glucose-containing chemically defined medium [Terleckyj et al., 1975]. The bacteria were washed, centrifuged at 4 °C and 18,000 *g* for 15 min, the supernatant discarded, and the pellet used for this test. Slabs of bovine teeth, abraded by 100 ± 10 µm and varnished to leave enamel windows of approximately 2×2 mm, were individually placed in 0.8 ml capped polypropylene tubes with the bacterial slurry. This was incubated with 5% (w/v) sucrose, 5% (w/v) sorbitol, or double-distilled deionized water in the ratio of 3 µl substrate/mg wet weight bacteria at 37 °C for 24 h with continuous shaking. The enamel slabs were placed in fresh bacterial slurry and substrate each 24 h for 4 days [Primrose et al., 1987]. The pH value was measured and 30-µl aliquots taken for acid anion and calcium analyses at various intervals. These aliquots were centrifuged as above and the supernatant used for the analyses. The acid anions (formate, pyruvate, lactate, succinate, acetate, and propionate) were analyzed using isotachophoresis [Geddes and Weetman, 1981] and the calcium assayed colorimetrically with a modification of the Sigma diagnostic kit using *o*-cresolphthalein complexone, read at 575 nm.

At the end of the 4 days, the enamel slabs were sectioned perpendicular to the surface and hand ground to approximately 100 µm. The exact thickness was measured along the length of the section using a digital micrometer and the similarity of the measurements checked to ensure that the sections were planoparallel. The sections were then mounted on Kodak high-resolution plates (type 1A) and microradiographed using $CuK_{\alpha}Ni$-filtered radiation (30 mA, 20 kV, 300 mm target focus distance) for 20 min. Microdensitometry was performed using a Leitz/ASBA image analyzer to quantify mineral content [Strang et al., 1987].

Table 1. pH at various time intervals for each substrate (mean ± SD; n = 16)

Time	Sucrose	Sorbitol	Water
0 min	5.2 ± 0.3	5.2 ± 0.4	5.3 ± 0.4
10 min	4.7 ± 0.2	5.0 ± 0.2*	5.2 ± 0.2*
30 min	4.2 ± 0.2	5.1 ± 0.2*	5.2 ± 0.2*
1 h	4.0 ± 0.1	5.1 ± 0.2*	5.1 ± 0.1*
5 h	3.8 ± 0.1	5.1 ± 0.1*	5.3 ± 0.1*
24 h	4.1 ± 0.1	5.2 ± 0.2*	5.5 ± 0.2*

* $p < 0.001$ compared to the value for 5% (w/v) sucrose (Student's t-test).

Table 2. Total acid anions (nmol/mg wet weight of bacteria) for each substrate (mean ± SD; n = 16)

Time	Sucrose	Sorbitol	Water
0 min	19.9 ± 3.4	19.7 ± 3.6	17.6 ± 4.8
10 min	23.2 ± 4.5	17.2 ± 2.4	15.4 ± 4.8*
30 min	32.6 ± 5.1	19.6 ± 2.9*	16.3 ± 2.2*
1 h	47.4 ± 6.3	21.9 ± 3.6*	18.6 ± 1.6*
5 h	92.5 ± 17.3	32.1 ± 5.6*	24.8 ± 2.7*
24 h	163.1 ± 19.5	47.7 ± 6.5*	37.5 ± 7.1*

* $p < 0.001$ compared to the value for 5% (w/v) sucrose (Student's t-test).

Table 3. Calcium release (m*M*) for each substrate (mean ± SD; n = 16)

Time	Sucrose	Sorbitol	Water
0 h	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.2
24 h	7.0 ± 3.9	0.4 ± 0.5*	0.5 ± 0.6*

* $p < 0.001$ compared to the value for 5% (w/v) sucrose (Student's t-test).

The sucrose, sorbitol, and water 4-day tests were each run four times, giving 16 24-hour sets of pH, acid anion, and calcium results and 4 sets of tooth slabs for microdensitometric analysis.

With 5% (w/v) sucrose, the pH fell rapidly (table 1), and the total identifiable acid anion concentration rose concomitantly over the 24-hour test period (table 2). The falls in pH (table 1) for the 5% (w/v) sorbitol and water were slight, as were the increases in acid anion concentrations (table 2). These differences between sucrose and both sorbitol and water were highly significant ($p < 0.001$) using a Student's t-test. There were no significant differences between the sorbitol and water controls.

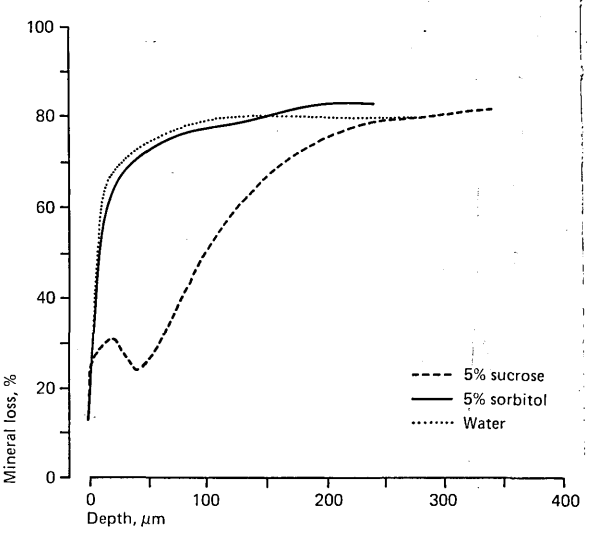

Fig. 1. Graph of % volume mineral loss against lesion depth for abraded bovine enamel incubated with 5% sucrose, 5% sorbitol, and water. Example of each condition.

The calcium concentration (table 3) increased with sucrose and showed little or no increase with sorbitol and water. Again the differences between sucrose and the controls were highly significant ($p < 0.001$) and non-significant between sorbitol and water. The high standard deviation values in the calcium analyses are the result of pooling the results over the 4 consecutive days of the experiment. The amount of calcium released from the slabs increased daily over the 4 days, reflecting increasing demineralization. The mineral profiles clearly show differences in the demineralizing potential of sucrose and both sorbitol and water, and an example of each is given in figure 1. In each case sucrose showed considerably more demineralization than sorbitol or water. Due to the small number of microdensitometric analyses obtained from the slabs, statistical analysis was not appropriate.

These results show that this test is capable of differentiating substances of known cariogenic potential. It should, therefore, be possible to test foods of interest and grade them as having a cariogenic potential: greater than or equal to that of sucrose, less than or equal to that of sorbitol, or between that of sucrose and

sorbitol. Foods in this last category should be considered for further testing in the human plaque pH and/or animal caries tests.

In this test, the biological variability resulting from differences in oral clearance, salivary flow, and unabraded enamel was excluded in order to gain reproducibility, and, therefore, the absence of these variables needs to be borne in mind when interpreting the results. The test system described in this paper does not incorporate amylase; however, incorporation of this enzyme would ensure appropriate conditions for estimation of the cariogenic potential of starchy foods. Amylase incorporation is the subject of further investigation; this is preferred to the incorporation of human saliva in order to maintain the reproducibility of the test.

The advantages of this model are that it is relatively inexpensive, rapid, and requires no highly specialized skills to perform. The ratios of bacteria:enamel:substrate are comparable to those found orally. The system is adaptable, and it is not limited to individual foodstuffs. It gives information on many parameters implicated in caries, i.e., pH, acid anions, calcium, phosphate, and fluoride (can be assessed if it is present in the food to be tested), and microradiographic and microdensitometric data can be obtained. As it uses a single bacterial strain and abraded bovine enamel, it is also very reproducible.

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